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(54) Title: REPRESSORS OF THE TRANS-ACTIVATING FUNCTION OF PAPILLOMAVIRUS E2 PROTEINS

PRO VAL ASP LEU ALA SER ARG GLN GLU GLU GLU GLU GLN
285 290

SER PRO ASP SER ^{Ser} **THR** GLU GLU GLU PRO VAL THR LEU PRO
300 310

ARG ARG THR THR ASN ^{Ile} **ASP** GLY PHE HIS LEU LEU LYS ALA
320

GLY GLY SER CYS PHE ALA LEU ILE SER ^{Val} **GLY** THR ALA ASN
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^{Ile} **GLN** VAL ^{met} **CYS** ^{S.R.O.F.Y} TYR ARG PHE ^{Ile} **ARG** VAL LYS LYS ^{Ile} **ASN** ^{Ile} **HIS**
340

ARG HIS ARG TYR GLU ASN CYS THR THR THR ^{Ser} **TRP** PHE THR
350 360

VAL ALA ASP ^{Ile} **ASN** GLY ALA GLU ^{Ile} **ARG** GLN GLY ^{Ile} **GLN** ^{Ser} **ALA** ^{Ile} **GLN**
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^{Ile} **ILE** LEU ILE THR PHE GLY SER PRO SER GLN ^{Arg} GLN ASP
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PHE LEU ^{Ile} **LYS** IIS VAL PRO LEU PRO PRO ^{Val} **GLY** ^{Ile} **MET** ASN ILE
390 400

SER GLY PHE THR ALA SER **LEU** **ASP** **PHE** **END** ^{Ile - 22 a.a.} ^{Ile - 22 a.a.}
410

(57) Abstract

This invention relates to E2 *trans*-activation repressors which interfere with normal functioning of the native full-length E2 transcriptional activation protein of the papillomavirus. Native full-length E2 *trans*-activation protein activates transcription of papillomavirus only through binding to DNA, and it binds to DNA only in the form of a pre-formed homodimer -- a pair of identical polypeptide subunits held together by non-covalent interactions. The E2 *trans*-activation repressors of this invention are proteins, polypeptides or other molecules that dimerize with full-length native E2 polypeptides to form inactive heterodimers, thus interfering with the formation of active homodimers comprising full-length native E2 polypeptides, thereby repressing papillomavirus transcription and replication. The E2 *trans*-activation repressors of this invention are advantageously used in the treatment of papillomavirus infections and their associated diseases.

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REPRESSORS OF THE TRANS-ACTIVATING FUNCTION OF
OF PAPILLOMAVIRUS E2 PROTEINS

TECHNICAL FIELD OF INVENTION

This invention relates to E2 trans-activation
5 repressors which interfere with normal functioning of
the native full-length E2 transcriptional activation
protein of the papillomavirus. Native full-length E2
trans-activation protein activates transcription of
papillomavirus only through binding to DNA, and it
10 binds to DNA only in the form of a pre-formed
homodimer -- a pair of identical polypeptide subunits
held together by non-covalent interactions. The E2
trans-activation repressors of this invention are
proteins, polypeptides or other molecules that dimerize
15 with full-length native E2 polypeptides to form
inactive heterodimers, thus interfering with the
formation of active homodimers comprising full-length
native E2 polypeptides, thereby repressing
papillomavirus transcription and replication. The E2
20 trans-activation repressors of this invention are
advantageously used in the treatment of papillomavirus
infections and their associated diseases.

BACKGROUND ART

Papillomaviruses are a group of small DNA
25 viruses that cause disease and pathological conditions
in animals and humans. These tumorigenic viruses

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produce benign tumors or lesions which may, in some instances, develop into malignancies. Papillomaviruses have been implicated as a cause of cervical cancer, as well as other anogenital and epithelial malignancies.

5 Papillomaviruses consist of icosahedral particles containing protein and a single, circular, double-stranded DNA molecule averaging 7.8 kbp. To date, more than ten animal papillomaviruses and more than fifty-five human papillomaviruses have been
10 identified (R. Sousa et al., "Control of Papillomavirus Gene Expression", Biochimica et Biophysica Acta, 1032, pp. 19-37 (1990); E.M. DeVilliers, "Heterogeneity of the Human Papillomavirus Group", J. Virol., 63, pp. 4898-903 (1989)). One particularly studied
15 papillomavirus is bovine papillomavirus ("BPV").

All known papillomaviruses encode similar proteins that perform analogous functions in infected cells. The E2 transcriptional activation protein ("the E2 protein") is a trans-acting factor that activates
20 transcription through specific binding to cis-acting E2 enhancer sequences (i.e., E2 binding sites) in viral DNA (E.J. Androphy et al., "Bovine Papillomavirus E2 Trans-Activating Gene Product Binds to Specific Sites in Papillomavirus DNA", Nature, 324, pp. 70-73 (1987)).
25 The 410 amino acid papillomavirus E2 protein has been shown to induce promoter expression in a classical enhancer mechanism (B.A. Spalholz et al., "trans-activation of a Bovine Papilloma Virus Transcriptional Regulatory Element by the E2 Gene Product", Cell, 42,
30 pp. 183-91 (1985)). As with other transcription factors, the functions of E2 protein appear to be localized to discrete modular domains (I. Giri and M. Yaniv, "Structural and Mutational Analysis of E2
Trans-Activating Proteins of Papillomaviruses Reveals

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Three Distinct Functional Domains", EMBO J., 7,
pp. 2823-29 (1988)).

- Papill mavirus infections are non-lytic in their natural hosts, indicating that transcription and replication of the papillomavirus are tightly controlled. An upstream regulatory region ("URR") is found immediately 5' to the early genes of BPV and other papillomaviruses. The URR contains cis-acting regulatory signals, including an origin of DNA replication and several promoters that function in early gene transcription. The URR also contains enhancer elements that activate transcription from the URR promoters and heterologous promoters (Sousa et al., supra).
- The E2 enhancer elements are conditional, in that they stimulate transcription only when activated by a protein encoded by a papillomavirus E2 open reading frame ("ORF"). Gene products from the E2 ORF include the full-length transcriptional activator E2 protein and at least two truncated versions of the E2 protein in BPV1 that function as transcriptional repressors. Transcriptional activation and repression of viral genes by E2 gene products constitute critical regulatory circuits in papillomavirus gene expression and DNA replication. E2 genes and DNA binding sites for E2 gene products appear to be characteristic of all papillomaviruses, although placement of the binding sites may vary Id.
- Transcriptional regulation by the E2 protein depends on its direct binding to the nucleotide sequence 5'ACCXNNNNYGGT3', which is found within cis-acting E2 enhancer elements in all papillomaviruses (Androphy et al., supra; Dartmann et al., "The Nucleotide Sequence and Genome Organization of Human Papilloma Virus Type 11", Virology, 151, pp. 124-30

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- (1986); H. Hirochika et al., "Enhancers and Trans-Acting E2 Transcriptional Factors of Papill mavirus", J. Virol., 61, pp. 2599-606 (1987); P. Hawley-Nelson et al., "The Specific DNA Recognition Sequence of the
- 5 Bovine Papillomavirus E2 Protein is an E2-Dependent Enhancer", EMBO J., 7, pp. 525-31 (1988); A.A. McBride et al., "The Carboxy-Terminal Domain Shared by the Bovine Papillomavirus E2 Transactivator and Repressor Proteins Contains a Specific DNA Binding Activity",
- 10 EMBO J., 7, pp. 533-39 (1988)). In that sequence, N represents any nucleotide; X is any nucleotide -- but is usually G, and Y represents any nucleotide -- but is usually C. E2 binding sites appear to be positioned in close proximity to the viral promoters, with seventeen
- 15 E2 binding sites being present throughout the bovine papillomavirus genome (R. Li et al., "Specific Recognition Nucleotides and their DNA Context Determine the Affinity of E2 Protein for 17 Binding Sites in the BPV-1 Genome", Genes Dev., 3, pp. 510-26 (1989)).
- 20 Enhancer elements containing E2 binding sites are found in the URR's of all papillomaviruses, as well as in other sites near promoters throughout the viral genome.

E2 binding sites may function as an element in viral DNA replication, as well as a classical transcriptional enhancer element. E2-mediated DNA binding, therefore, is essential for the natural life cycle of papillomaviruses.

European patent application 302,758 refers to the use of modified forms of E2 protein that bind to, and block, E2 binding sites on papillomavirus DNA without resulting in trans-activation. That application also refers to repression of E2 activation through the use of DNA fragments that mimic E2 binding sites, and thus bind with E2 trans-activators, making

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them unavailable for binding to E2 sites in the viral DNA.

E2 protein also binds the papillomavirus replication protein known as E1. It has been proposed that when an E2/E1 complex binds to an E2 binding site, replication of the viral genome occurs (M. Botchan et al., International Papillomavirus Workshop, Heidelberg, Germany (May 1990); Mohr et al., "Targeting the E1 Replication Protein to the Papillomavirus Origin of Replication by Complex Formation with the E2 Transactivator", Science, 250, pp. 1654-99 (1990)).

Full-length E2 transcriptional activator polypeptides (monomers) have a molecular weight of about 50 kD. Although amino acid sequence homology among E2 proteins of various papillomaviruses is low (ca. 35%), the E2 proteins share conserved motifs that constitute unique structural domains having distinct functions (Giri and Yaniv, supra).

The C-terminal domain of the E2 polypeptide is responsible for recognition of E2 binding sites on viral DNA. The N-terminal domain of the E2 polypeptide is responsible for transcriptional activation following binding of the protein to viral DNA (A.A. McBride et al., "E2 Polypeptides Encoded by Bovine Papillomavirus Type 1 Form Dimers Through The Common Carboxyl-Terminal Domain: trans-Activation is Mediated by the Conserved Amino Terminal Domain", Proc. Natl. Acad. Sci. USA, 86, pp. 510-14 (1989)). The E2 protein binds to viral DNA in vivo only in the form of a pre-existing homodimer Id. Dimeric E2 proteins exert control of papillomavirus promoters by directly binding to an inverted repeat that has been found in all such viruses.

In bovine papillomavirus models, and in some human papillomaviruses, at least two N-terminally

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truncated E2 proteins occur naturally and act as native repressors. It has been experimentally confirmed in vitro that truncated forms of E2 proteins which retain their ability to bind DNA but do not trans-activate, 5 are competitive inhibitors of trans-activation-competent E2 polypeptides (P.F. Lambert et al., "A Transcriptional Repressor Encoded By BPV-1 Shares A Common Carboxy-Terminal Domain With The E2 Transactivator", Cell, 50, pp. 69-78 (1987); 10 A. Stenlund and M.R. Botchan, "The E2 Trans-Activator Can Act as a Repressor by Interfering with a Cellular Transcription Factor", Genes Dev., 4, pp. 123-36 (1990); J. Choe et al., "Bovine Papillomavirus Type 1 Encodes Two Forms of a Transcriptional Repressor: 15 Structural and Functional Analysis of New Viral cDNAs", J. Virol., 63, pp. 1743-55 (1989)). That inhibition has never been definitively attributed to competition for DNA binding sites, for E2 polypeptides in the dimerization process, or for both. It has been 20 suggested that transcriptional repression occurs through direct competition with the native full-length, i.e., transcriptionally active E2 protein at the DNA binding site. PCT patent application WO89/12461 refers to peptide inhibitors of viral gene expression and 25 viral replication. Those inhibitors are said to bind to trans-activator binding sites in viral DNA, thus blocking normal binding of native trans-activating proteins to those sites. And it has been suggested that formation of non-functional protein complexes 30 could also prevent E2 activation of transcription (P.F. Lambert et al., supra).

Although it is known that papillomavirus E2 protein is the sequence-specific DNA binding protein that coordinates papillomavirus transcription, the 35 structures of its DNA binding and dimerization motifs

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hav never been determined. Both th DNA binding activity and the dim rizati n signal of the papillomavirus E2 trans-activation prot in reside in the carboxy terminal 100 amino acids of the prot in 5 (McBride et al., supra). The C-terminal 100, 125 or 249 amino acids of E2 protein (each of which lacks trans-activation activity) all repress E2-dependent gene expression (T. Haugen et al., "Sequence-Specific and General Transcriptional Activation by the Bovin 10 Papillomavirus-1 E2 Trans-Activator Require an N-Terminal Amphipathic Helix-Containing E2 Domain", EMBO J., 7, pp. 4245-53 (1988)). Although the capacity for E2 dimerization, as well as the capacity for site-specific DNA binding, are known to reside in the C-terminal domain of the E2 polypeptide, the amino acid 15 region within that domain responsible for E2 dimerization has not been identified (Giri and Yaniv, supra). To date, the dimerization function of the E2 polypeptide has not been separated from its DNA-binding 20 function. Accordingly, repressors that inhibit papillomavirus transcription and replication by interfering with dimerization of native full-length E2 polypeptides have remained unknown.

DISCLOSURE OF THE INVENTION

25 By virtue of the present invention, the dimerization function of the E2 polypeptide has been separated from its DNA binding function. That separation has enabled, for the first time, the production of E2 trans-activation repressor 30 polypeptides that are homologous to papillomavirus E2 polypeptides and which inhibit transcription and replication of papillomaviruses by interfering with dimerization of native full-length E2 polypeptides. These E2 trans-activation repressors advantageously

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exert their anti-viral effects by interfering with E2-protein-mediated enhancement of papillomavirus transcription in cells infected with that virus. The E2 trans-activation repressors of this invention are characterized by their ability to form inactive E2 heterodimers with full-length native E2 polypeptides produced by the papillomavirus and, therefore, to interfere with the formation of active homodimers by those polypeptides. By virtue of those abilities, the E2 trans-activation repressors reduce the availability of full-length native E2 polypeptides for formation of active homodimers, thus repressing papillomavirus transcription and replication.

According to one embodiment of this invention, E2 trans-activation repressors comprise at least the dimerization region, but less than the DNA binding domain, of the E2 polypeptide. Such repressors, which interfere with DNA binding by full-length E2 polypeptides through formation of inactive heterodimers and which comprise less than the DNA binding domain of the E2 polypeptide, by virtue of their reduced size, advantageously reduce the potential problem of repressor uptake into papillomavirus-infected cells. These repressors are useful in processes and compositions for treating papillomavirus infections.

This invention also relates to methods for isolating mutations in DNA encoding polypeptides that are homologous to native E2 polypeptides and which form inactive heterodimers with native full-length E2 polypeptides. Such mutations are useful in processes and compositions for the treatment of papillomavirus infections.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the amino acid sequence of a segment of the wild type BPV1 E2 polypeptide between amino acids 285 and 410, which is the "DNA binding domain". Rectangles (and abbreviations immediately above the rectangles) indicate changes made in that amino acid sequence to produce E2 mutants (homologues), including the E2 trans-activation repressors of this invention.

10 Figure 2 tabulates the DNA binding, dimerization and repression activities of various E2 mutants prepared according to this invention.

Figure 3 shows autoradiograms of electrophoresis gels from DNA binding ("gel shift") assays.

15 Figure 4 shows autoradiograms of electrophoresis gels from a super-shift DNA binding assay performed with dimerization-defective mutant polypeptide 360S.

20 Figure 5 schematically depicts the construction of plasmid pXB332.

Figure 6 schematically depicts the construction of plasmid pXB323hGH.

25 Figure 7 schematically depicts the construction of plasmid pXB101.

Figure 8 schematically depicts the construction of plasmid pXB323.

Figure 9 schematically depicts the construction of plasmid pXB314.

30 Figure 10 schematically depicts the construction of plasmids pEC337L, pEC339M, pEC340F, pEC340R, pEC340Y and pEC344L.

Figure 11 schematically depicts the construction of plasmids pBG331 and pAHE2.

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Figure 12 schematically depicts the construction of plasmid pET8c-85.

Figure 13 schematically depicts the construction of plasmid pHE2-85.

5 Figure 14 schematically depicts the construction of plasmid pET8c-123.

Figure 15 schematically depicts the construction of plasmid pHE2-123.

10 Figure 16 schematically depicts the construction of plasmid pFTE501.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

15 In the description, the following terms are employed:

E2 trans-Activation Repressor -- Any protein, polypeptide or other molecule that interferes with transcriptional activation of papillomavirus resulting 20 from binding of full-length native E2 polypeptides in the form of pre-existing homodimers to E2 binding sites on DNA.

DNA Binding Domain -- Amino acids 285-410 of BPV1 E2 protein, or the corresponding homologous region 25 of an HPV E2 protein.

Native Minimal DNA Binding Domain -- Amino acids 325-410 of BPV1 E2 protein, or amino acids 283-365 of HPV16 E2 protein, each amino acid sequence being sufficient for dimerization and binding to E2 DNA 30 binding sites.

Homologous -- An amino acid sequence very similar to at least a portion of the "DNA binding domain" but having at least one mutation therefrom, or a nucleic acid sequence encoding an amino acid sequence

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very similar to at least a portion of the "DNA binding domain" but having at least one mutation therefrom.

Homologue -- A polypeptide or nucleic acid that is homologous to a native E2 polypeptide or a native E2 gene, respectively.

Mutant -- Homologue or homologous.

Mutation -- A substitution, insertion or deletion in a gene encoding a desired protein or polypeptide.

10 E2 Protein Dimerization Region -- The region of the DNA binding domain that is necessary and sufficient for dimerization but not sufficient for binding of the dimer resulting from that dimerization to DNA.

15 Transport Moiety -- Any covalent addition to an E2 trans-activation repressor that facilitates entry of that repressor into target cells.

20 Inactive Heterodimers -- Dimers that comprise two non-identical polypeptide subunits and which do not bind to E2 binding sites on DNA.

Active homodimers -- Dimers that comprise two identical E2 polypeptide subunits held together by non-covalent interactions and which cause transcriptional activation upon binding to E2 DNA binding sites.

25 Reporter Gene -- A gene whose expression depends on the occurrence of a cellular event of interest and can be conveniently observed in a genetically transformed host strain.

30 Reporter Plasmid -- A plasmid vector that comprises one or more reporter genes.

Reporter Strain -- A genetically transformable unicellular host strain that comprises one or more reporter plasmids.

35 Amino Acid -- A monomeric unit of a peptide, polypeptide or protein. The twenty protein amino acids

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(L-isomers) are: phenylalanine ("Phe" or "F"), leucine ("Leu", "L"), isoleucine ("Ile", "I"), methionine ("Met", "M"), valine ("Val", "V"), serine ("Ser", "S"), proline ("Pro", "P"), threonine ("Thr", "T"), alanine ("Ala", "A"), tyrosine ("Tyr", "Y"), histidine ("His", "H"), glutamine ("Gln", "Q"), asparagine ("Asn", "N"), lysine ("Lys", "K"), aspartic acid ("Asp", "D"), glutamic acid ("Glu", "E"), cysteine ("Cys", "C"), tryptophan ("Trp", "W"), arginine ("Arg", "R") and glycine ("Gly", "G").

As set forth in the examples of this application, E2 trans-activation repressors may be produced by random mutations and site-directed mutations in the C-terminal, 126-residue DNA binding domain of the E2 gene. Those mutations yielding functionally defective mutants or homologues of E2 polypeptides may be isolated following transformation of a unicellular host strain carrying an E2 trans-activation reporter plasmid. The isolated mutations may then be analyzed in terms of:

- a) expression of a protein that is recognized by E2 antibodies and that has approximately the molecular weight (50 kD) expected for a full-length native E2 polypeptide;
- b) nucleotide sequence of the mutated region of the E2 gene -- the region of the E2 gene that encodes the DNA binding domain -- which is in the C-terminal region of the E2 polypeptide;
- c) capacity of the mutant (homologous) polypeptide corresponding to the C-terminal region of the native E2 polypeptide, to bind to E2 DNA binding sites;
- d) capacity of the mutant (homologous) polypeptide corresponding to the C-terminal region of the native E2 polypeptide, to dimerize with itself;

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e) capacity of the mutant (homologous) polypeptide corresponding to the C-terminal region of the native E2 polypeptide, to repress E2-dependent trans-activation in eukaryotic cells.

5 By virtue of this invention, for the first time, the E2 protein dimerization region -- the region of the amino acid sequence in the E2 DNA binding domain that is responsible for dimerization independent of DNA binding interactions -- was located between amino acids
10 325 and 410 of the native E2 protein. In addition, it was recognized that the E2 protein dimerization region itself is sufficient to repress trans-activation by full-length E2 proteins and that DNA binding is not required for repression of E2 trans-activation.
15 Further, it was discovered that several mutations in the E2 amino acid sequence abolish DNA binding without abolishing dimerization.

According to one embodiment of this invention, an E2 trans-activation repressor comprises a
20 polypeptide having an amino acid sequence homologous to the E2 DNA binding domain (SEQ ID NO:1), or homologous to a polypeptide fragment thereof, said polypeptide being capable of forming inactive heterodimers with the full-length native E2 polypeptides produced by the
25 papillomavirus and said inactive heterodimers being incapable of binding to E2 DNA binding sites.
Alternatively, an E2 trans-activation repressor of this invention consists essentially of a polypeptide having an amino acid sequence homologous to the native E2 DNA
30 binding domain (SEQ ID NO:1), or homologous to a polypeptide fragment thereof, said polypeptide being capable of forming inactive heterodimers with the full-length native E2 polypeptide and said inactive heterodimers being incapable of binding to E2 DNA
35 binding sites.

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In another embodiment of this invention, an E2 trans-activation repressor comprises a polypeptide that is a fragment of the native E2 DNA binding domain. Alternatively, an E2 trans-activation repressor of this 5 invention consists essentially of a polypeptide that is a fragment of the native E2 DNA binding domain.

In another embodiment of this invention, an E2 trans-activation repressor comprises an amino acid sequence selected from the group consisting of the 10 amino acid sequence defined by SEQ ID NO:3, the amino acid sequence defined by SEQ ID NO:5, the amino acid sequence defined by SEQ ID NO:7, the amino acid sequence defined by SEQ ID NO:9, the amino acid sequence defined by SEQ ID NO:11, the amino acid 15 sequence defined by SEQ ID NO:13, the amino acid sequence defined by SEQ ID NO:15, the amino acid sequence defined by SEQ ID NO: 23, and the amino acid sequence defined by SEQ ID NO: 25. E2 trans-activation repressors of this invention may also consist 20 essentially of any one of those amino acid sequences.

It should be understood that this invention also relates to E2 trans-activation repressors other than those defined by SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID 25 NO:15, SEQ ID NO: 23 and SEQ ID NO: 25. More particularly, E2 trans-activation repressors according to this invention include polypeptides comprising fragments of the E2 DNA binding domain or amino acid sequences homologous to the E2 binding domain, so long 30 as those polypeptides demonstrate the capacity to repress E2 trans-activation by interfering with formation of active E2 homodimers.

The E2 trans-activation repressors of this invention may be chemically synthesized by conventional 35 peptide synthesis techniques, such as solid phase

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synth sis (R.B. Merrifield, "Solid Phas Peptide Synth sis.I. Th Synth sis Of A T trapeptide", J. Am. Chem. Soc., 83, pp. 2149-54 (1963)). Alternatively, they may be prepared in appropriate hosts transformed
5 with DNA sequences that code for the desired E2 trans-activation polypeptide. For example, an E2 trans-activation repressor of this invention may be prepared in a process comprising the steps of:
10 a) culturing appropriate hosts that have been transformed with and which express a DNA sequence encoding that polypeptide; and b) recovering the E2 trans-activation repressor from the culture.

E2 trans-activation repressors according to this invention may also be produced by truncating a
15 full-length native E2 gene, or a portion thereof, at various positions to encode a polypeptide that is a fragment of the native E2 binding domain and that comprises the E2 dimerization region, but lacks sequences necessary for DNA binding. For example, a
20 papillomavirus E2 gene may be truncated so as to encode a polypeptide consisting of a sequence beginning between about amino acid 338 and amino acid 360, and ending at about amino acid 410. Such truncation of the full-length native E2 gene, or a portion thereof, may
25 be accomplished by conventional techniques involving restriction digestion and oligonucleotide linkers, or by exonuclease digestion. A combination of such methods may also be employed to design E2 repressors other than those illustrated herein.

30 When an E2 trans-activation repressor of this invention is produced by expression in a unicellular host transformed with a DNA sequence encoding the repressor, the DNA sequence should be operatively linked to an expression control sequence in an
35 appropriate expression vector and employed in that

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vector to transf rm an appropriate unic llular host. Such operativ linking of a DNA sequ nce encoding an E2 trans-activation repressor of this invention to an expression control sequence, of course, includes th 5 provision of a translation start signal in the correct reading frame upstream of that DNA sequence. If the particular DNA sequence to be expressed does not begin with a methionine, the start signal will result in an additional amino acid -- methionine -- being located at 10 the N-terminus of the product. While such methionyl-containing E2 trans-activation repressors may be employed directly in the compositions and methods of this invention, it is usually more desirable to r move the methionine before use. Methods are available in 15 the art to remove such N-terminal methionines from polypeptides expressed with them. For example, c rtain hosts and fermentation conditions permit removal of substantially all of the N-terminal methionine in vivo. Other hosts require in vitro removal of the N-terminal 20 methionine. Such in vivo and in vitro methods are well known in the art.

A wide variety of host/expression vector combinations may be employed in expressing DNA sequences encoding the E2 trans-activation repressors 25 of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E.coli, including col E1, 30 pCR1, pBR322, pMB9, pET-3A and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs, e.g., the numerous derivatives of phage λ , e.g., NM989, and other DNA phag s, .g., M13 and filamentous single-stranded DNA phages, yeast plasmids, such as the 2μ plasmid or 35 derivatives thereof, and vectors derived from combi-

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nations of plasmids and phage DNAs, such as plasmids which hav be n modifi d t employ phage DNA or other expression contr l sequenc s. For animal c ll expression, we prefer to use plasmid pJOD, which

5 contains the adenovirus major late promoter augm nted by the presence of the SV40 enhancer (J. Barsoum, "Introduction of Stable High Copy Number DNA into Chinese Hamster Ovary Cells by Electroporation", DNA and Cell Biol., 9, pp. 293-300 (1990)).

10 In addition, any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence when operatively linked to it -- may be used in these vectors to xpress DNA sequences encoding the E2 trans-activation
15 repressors of this invention. Such useful expr ssion control sequences, include, for example, the early and late promoters of SV40, adenovirus or cytomegal virus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whos
20 expression is directed by T7 RNA polymerase, th major operator and promoter regions of phage λ, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the
25 promoters of the yeast α-mating factors, the polyh dron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. For animal cell expression, w
30 prefer to use an expression control sequence derived from the adenovirus major late promoter augmented by the presence of the SV40 enhancer.

A wide variety of unicellular host cells are also useful in expressing DNA sequences encoding the E2
35 trans-activation repressors of this invention. These

hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E.coli, Pseudomonas, Bacillus, Streptomyces, Saccharomyces and other fungi, animal cells, such as Chinese hamster ovary ("CHO") and 5 mouse cells in culture, African green monkey cells, such as COS 1, COS 7, BSC 1, BSC 40, and BMT 10, insect cells in culture, human cells in culture and plant cells in culture. For animal cell expression, we prefer CHO cells.

10 It should of course be understood that not all vectors and expression control sequences will function equally well to express DNA sequences encoding the E2 trans-activation repressors of this invention. Neither will all hosts function equally well with the 15 same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation and without departing from the scope of this invention. For example, in selecting a vector, 20 the host must be considered, as the vector must replicate in it. The vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

25 In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the system, its controllability and its compatibility with the DNA sequence encoding the particular E2 30 trans-activation repressor of this invention, particularly with respect to potential secondary structures. Unicellular hosts should be selected by consideration of their compatibility with the chosen vector, any potential toxicity of the product coded for 35 on expression by the DNA sequences of this invention to

them, their secretion characteristics, their ability to fold proteins correctly, their fermentation requirements and the ease of purification of the products coded for on expression by DNA sequences 5 encoding the particular E2 trans-activation repressor of this invention.

The E2 trans-activation repressor polypeptides produced on expression of the DNA sequences of this invention may be isolated from the 10 fermentation or animal cell cultures and purified using any of a variety of conventional methods. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

15 E2 trans-activation repressors according to this invention also include non-peptide chemicals -- peptidomimetics -- which are capable of specifically forming an inactive complex with native full-length E2 polypeptides so as to prevent them from forming active 20 homodimers and thereby blocking papillomavirus transcription and translation. And molecules that form a stable complex with E2 polypeptides so as to prevent them from forming active homodimers may be designed on the basis of 3-dimensional data on the E2 dimerization 25 domain. Three-dimensional data on the E2 dimerization domain may be obtained by X-ray crystallography.

The structural motif represented in BPV1 by amino acids 333 through 344 of the E2 protein is highly conserved among papillomaviruses, including human 30 papillomaviruses (Giri and Yaniv, supra). Several papillomavirus E2 repressors of this invention comprise mutations in that highly conserved motif. It should be understood that the BPV1-derived E2 trans-activation repressors of this invention are useful in the 35 treatment of human papillomavirus infections. It

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should be further understood that the illustrative processes for the production of E2 trans-activation repressors from the bovine papillomavirus, BPV1, described in this application, may similarly be
5 employed to produce E2 trans-activation repressors from human papillomaviruses, as demonstrated in Example 7.

The processes and compositions of this invention may be used to treat any mammal, including humans. According to this invention, mammals are
10 treated by the pharmaceutically acceptable administration of an E2 trans-activation repressor in a pharmaceutically effective amount and for a period of time sufficient to inhibit or lessen the spread of papillomavirus infection, to reduce the symptoms of the
15 specific papillomavirus-associated disease, or to prevent their recurrence.

Diseases which may be treated by the processes and compositions of this invention are those caused by the etiological agent, papillomavirus. Such
20 diseases include, for example, epithelial malignancies, anogenital malignancies, such as cervical cancer, malignant lesions, benign lesions, papillomacarcinomas, papilloadenocystomas, papilloma neurophathicum, papillomatosis, cutaneous and mucosal papillomas,
25 condylomas, oral, pharyngeal, laryngeal, and tongue papillomas, fibroblastic tumors and other pathological conditions associated with papillomavirus. The E2 trans-activation repressors of this invention may also be used to treat epithelial and internal
30 fibropapillomas in animals. In addition, the methods and compositions of this invention may be used for the recidivism prophylaxis of solid tumors.

According to this invention, E2 trans-activation repressors may be in any pharmaceutically
35 acceptable dosage form, including those which may be

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administered intratum rally, peritumorally, interlesionally, intravenously, intramuscularly, subcutaneously or perilesionally, or by topical routes, to exert local therapeutic effects.

- 5 Such dosage forms may include pharmaceutically acceptable carriers and adjuvants which are known to those of skill of the art. These carriers and adjuvants include, for example, ion exchangers, alumina, aluminum stearate, lecithin, serum 10 proteins, such as human serum albumin, buffer substances, such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium 15 hydrogen phosphate, potassium hydrogenphosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances and polyethylene glycol. Adjuvants for topical or gel base forms of E2 trans-activation 20 repressors may, for example, be selected from the group consisting of sodium carboxymethylcellulose, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol and wood wax alcohols. For all administrations, conventional depot forms may 25 be used.

The pharmaceutical compositions of this invention may be formulated using conventional methods to prepare pharmaceutically useful compositions. Such compositions preferably include at least one 30 pharmaceutically acceptable carrier. See, e.g., Remington's Pharmaceutical Sciences (E.W. Martin). In addition, the compositions preferably include a pharmaceutically acceptable buffer, preferably phosphate buffered saline, together with a 35 pharmaceutically acceptable compound for adjusting

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isotonic pressure, such as, for example, sodium chloride, mannitol or sorbitol.

Pharmaceutical compositions according to this invention may include one or more E2 trans-activation repressors as active ingredients. Alternatively, a composition containing one E2 trans-activation repressor may be administered to a patient in combination with, or sequentially with, a composition containing a different E2 trans-activation repressor.

The most effective mode of administration and dosage regimen of the E2 trans-activation repressor will depend upon the type of disease to be treated, the severity and course of that disease, previous therapy, the patient's health status and response to the E2 repressor and the judgment of the treating physician. The E2 repressor may be administered to the patient at one time or over a series of treatments.

According to one embodiment of this invention, papillomavirus-infected cells may be saturated with an E2 trans-activation repressor which forms inactive heterodimers with the native full-length E2 polypeptides produced by that virus, to interfere with the formation of active homodimers comprising native full-length E2 polypeptides, thus repressing viral transcription and replication.

Depending on the severity of the papillomavirus infection or its associated disease, for parenteral regimens, a dose of between about 1 and 1000 mg/kg body weight of the E2 trans-activation repressor may be administered to the patient, via one or several administrations, or released from a depot form per treatment. Alternatively, a dose of between about 1 and 1000 µg/ml of the E2 trans-activation repressor may be administered to a patient per application via topical routes.

According to an alternate embodiment of this invention, an E2 trans-activation repressor may be administered serially or in combination with other therapeutics used in the treatment of papillomavirus infections or diseases caused by them. Such therapeutics include interferons, such as IFN- γ , IFN- α and IFN- β derived from natural sources or produced by recombinant techniques, other cell mediators formed by leukocytes or produced by recombinant techniques such as for example, interleukin-1, interleukin-2, tumor necrosis factor, macrophage colony stimulating factor, macrophage migration inhibitory factor, macrophage activation factor, lymphotoxin and fibroblast growth factor. Alternatively, the E2 trans-activation repressor may be administered serially or in combination with conventional therapeutic agents or regimens such as, for example, salicylic acid, podophyllotoxin, retinoic acid, surgery, laser therapy and cryotherapy. Such combination therapies may advantageously utilize less than conventional dosages of those agents, or involve less radical regimens, thus avoiding any potential toxicity or risks associated with those therapies.

The E2 trans-activation repressors of this invention may be delivered to papillomavirus-infected cells either directly or indirectly. Direct delivery of E2 trans-activation repressors may be facilitated by chemical modification of the polypeptides themselves. One such modification involves increasing the lipophilicity of the E2 trans-activation repressor in order to increase binding to the cell surface, in turn, stimulating non-specific endocytosis of the protein. Lipophilicity may be increased by adding a lipophilic moiety (e.g., one or more fatty acid molecules) to the E2 repressor. A wide variety of fatty acids may be

employed. For example, the protein may be palmitoylated. Alternatively, a lipopeptid may be produced by fusion or cross-linking, to permit the E2 repressor to resemble the natural lipopeptide from 5 E.coli, tripalmitoyl-S-glycercylcystein-seryl-serin, at its amino terminus. This lipopeptide has been shown to increase the uptake of fused peptides (P. Hoffmann et al., "Stimulation Of Human And Murine Adherent Cells By Bacterial Lipoprotein And Synthetic Lipopeptid 10 Analogues", Immunobiol., 177, pp. 158-70 (1988)). Lipophilicity may also be increased by esterification of the protein at tyrosine residues or other amino acid residues. And uptake of the E2 trans-activation repressor may be increased by addition of a basic 15 polymer such as polyarginine or polylysine (W-C. Shih and H.J.P. Ryser, "Conjugation Of Poly-L-Lysine Albumin And Horseradish Peroxidase: A Novel Method Of Enhancing The Cellular Uptake Of Proteins", Proc. Natl. Acad. Sci USA, 75, pp. 1872-76 (1978)).

20 Because some uptake mechanisms for E2 trans-activation repressors may involve passage through lysosomes and since long half-life in the target cells is desirable, an E2 trans-activation repressor of this invention may be modified to increase its protease 25 resistance and, in turn, the half-life of the polypeptide in circulation and cells. In one embodiment of the present invention, the protease resistance of an E2 trans-activation repressor is increased by incorporation of D-amino acids instead of 30 L-amino acids at some or all residues of the polypeptide. In another embodiment, the amino terminus, or carboxy terminus, or both termini of an E2 repressor are blocked by chemical modification. In a further embodiment of this invention, lysosomal 35 proteases are inhibited by an E2 trans-activation

r pressor in a composition comprising a lysomotropic agent, such as chloroquin, amantadine, monensin, methylamine, or ammonium chloride.

Direct delivery of E2 trans-activation

- 5 repressors according to this invention may also be effected by the use of transport moieties, such as protein carriers known to cross cell membranes. For example, an E2 trans-activation repressor may be fused to a carrier protein, preferably by a genetic fusion 10 which may be expressed in a system such as E.coli or yeast. According to one embodiment of this invention, the amino terminus of the E2 trans-activation repressor may be fused to the carboxy terminus of a transport moiety using standard techniques.
- 15 Nucleotide sequences encoding such carrier-E2 trans-activation repressor fusion proteins, operatively linked to regulatory sequences, may be constructed and introduced into appropriate expression systems using conventional recombinant DNA procedures.
- 20 The resulting fusion protein may then be purified and tested for its capacity to (1) enter intact eukaryotic cells and (2) inhibit E2-dependent gene expression and viral DNA replication once inside the intact eukaryotic cells.
- 25 In choosing a useful carrier protein, those of skill in the art will recognize the desirability of appropriate control experiments designed, inter alia, to test the possibility that the carrier portion of the fusion protein itself interacts with elements of the E2 30 transcriptional regulation system. If the carrier portion of the fusion protein is found to have undesirable interactions, such as activation of E2-dependent enhancer elements, the portions of the carrier sequence responsible for these interactions 35 should be identified and deleted in a way which permits

the sequence to retain its carrier capacity.

Alternately, one of several conventional carrier sequences which do not interact with elements of the E2 transcriptional regulation system can be substituted.

5 Useful carrier proteins include, for example, bacterial hemolysins or "blending agents", such as alamethicin or sulfhydryl activated lysins. Other carrier moieties which may be used include cell entry components of bacterial toxins, such as Pseudomonas

10 exotoxin, tetanus toxin, ricin toxin, and diphtheria toxin. Also useful is melittin, from bee venom. Other useful carrier proteins include proteins which are viral receptors, cell receptors or cell ligands for

specific receptors that are internalized, i.e., those

15 which cross mammalian cell membranes via specific interaction with cell surface receptors, recognized and taken into the cell by cell surface receptors. Such cell ligands include, for example, epidermal growth factor, fibroblast growth factor, transferrin and

20 platelet-derived growth factor. Alternatively, the ligand may be a non-peptide, such as mannose-6-phosphate, which permits internalization by the mannose-6-phosphate receptor. The transport moiety may also be selected from bacterial immunogens, parasitic

25 immunogens, viral immunogens, immunoglobulins or fragments thereof that bind to target molecules, cytokines, growth factors, colony stimulating factors and hormones. A transport moiety may also be derived from the tat protein of HIV-1.

30 As an alternative or addition to the above-described chemical modifications and protein carriers, which may be employed alone or in combination, other agents which allow penetration of the keratinized cell layer may be employed to facilitate delivery of the E2
35 trans-activation repressors of this invention to

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papillomavirus-infected cells. In topical applications, for example, the E2 trans-activation repressor may be administered in combination with dimethylsulfoxide, an agent which promotes penetration of cell membranes by substances mixed with it. Useful keratinolytic agents include, for example, salicylic acid, urea, and α -hydroxyacids. For such applications, the E2 trans-activation repressor and any other agent may be administered topically, in cream or gel form.

10 Indirect delivery of an E2 trans-activation repressor to papillomavirus-infected cells may be carried out by delivering a gene encoding an E2 trans-activation repressor, with appropriate expression control sequences, into those cells. A gene encoding 15 an E2 trans-activation repressor may be introduced into target cells by treating the infected cells, for example, by scraping them to allow uptake of DNA, by electroporation, by direct injection, or through the use of defective recombinant viruses, such as 20 retroviruses. For example, a DNA sequence encoding an E2 trans-activation repressor may be introduced into target cells using a retrovirus by transcribing the DNA sequence encoding an E2 trans-activation repressor into an RNA sequence and incorporating the resulting RNA 25 sequence into a defective recombinant retrovirus.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not 30 to be construed as limiting this invention in any manner. Throughout these examples, all molecular cloning reactions were carried out according to methods in T. Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982) or 35 J. Sambrook et al., Molecular Cloning - A Laboratory

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Manual, Cold Spring Harbor Laboratory (1982) or J. Sambrook et al., Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press (1989), using enzymes obtained from New England Biolabs (Beverly, 5 Massachusetts), except where otherwise noted. We confirmed the integrity of all plasmid constructions by DNA sequencing.

EXAMPLE 1

10 Chemical Mutagenesis, Phenotypic Selection of Mutants And Site-Directed Mutagenesis

We cloned the full-length coding strand of the wild type BPV1 E2 gene from plasmid pCO-E2 (Hawley-Nelson et al., supra) into the filamentous single-stranded DNA bacteriophage M13 strain mp18 (Life 15 Technologies, Inc., Gaithersburg, MD) and isolated single-stranded DNA for chemical mutagenesis of the E2 protein DNA binding domain. See Chapter 4 of Sambrook et al., supra, for standard procedures pertaining to the use of bacteriophage M13.

20 In summary, in order to generate a large number of mutants, we chemically mutagenized and reverse-transcribed one strand of the BPV1 E2 gene, transferred the double-stranded segment into a wild type E2 yeast expression vector and isolated mutants 25 that were limited in gene induction. Random mutations in the DNA encoding the 126-residue C-terminal DNA binding domain of the E2 protein were produced by chemical mutagenesis, essentially according to the method of R.M. Myers et al., "A General Method for 30 Saturation Mutagenesis of Cloned DNA Fragments", Science, 229, pp. 242-47 (1985). The method of Myers et al., supra, involves brief exposure of single-stranded DNA to chemicals such as nitrous acid, formic acid, hydrazaine, or potassium permanganate, that

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damage all four bases without damaging the phosphodiester backbone of the DNA.

More specifically, we treated 20 µg of single-stranded M13 DNA containing the full-length E2 gene with 1.3 mM potassium permanganate for between about 5 and 10 min. In a variation of the procedure, we treated the single-stranded DNA with 12 M formic acid for between about 5 and 10 min. With either chemical reagent, we carried out the reaction at room temperature and stopped it by addition of 1/10 volume of 2.5 M sodium acetate (pH 7.0). We separated the chemically modified, single-stranded DNA from the reaction mixture by precipitating it twice with cold ethanol in the presence of yeast tRNA carrier. We further purified the chemically modified DNA by agarose gel electrophoresis.

For second strand synthesis, we annealed synthetic oligonucleotide primers complementary to the single-stranded DNA 3' to the BstX1 site which is at nucleotide 3881 in the 3' non-coding region of the BPV1 E2 gene. Any portion of this region may be used for priming, and the exact length of the primer is not critical -- so long as the primer is of a sufficient length to form a stable duplex. Conditions for annealing of primers and techniques of primer extension are well known in the art. We used a primer having the sequence 5' AGCAACTAGTCCCAAG 3', (SEQ ID NO:17) which is complementary to nucleotides 3904 to 3919 of BPV1. For primer extension, we used T7 polymerase (Sequenase 2.0, U.S. Biochemicals, Cleveland, Ohio). The primer extension reaction was carried out at 37°C for about 1 hr, in the presence of all four dNTPs, according to the vendor's recommendations. Alternatively, we used murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) at 40°C for

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primer extension. When the polymerase used for a second strand synthesis encountered a damaged base in the template strand, it incorporated any one of the four dNTPs. Random transitions and transversions, involving all four bases, were, therefore, likely to be produced at potentially any point in the nucleotide sequence. Thus, synthesis of the complementary DNA strand led to mutation at sites on the coding strand where chemical reaction took place.

10 We digested the primer extension products (i.e., double-stranded DNA) with restriction endonucleases KpnI and BstXI to release a population of 426 bp randomly mutagenized E2 gene fragments encoding the C-terminal region of the E2 protein.

15 We purified the mutagenized fragments on an agarose gel and subcloned them into the wild type E2 gene in yeast expression vector pYE2 (Morrissey et al., supra), replacing the corresponding wild type 426 bp KpnI-BstXI fragment with a mutagenized fragment.

20 Plasmid pYE2 comprises a galactose upstream activating sequence ("GAL UAS") and downstream restriction sites such that the GAL UAS can conveniently be used to drive expression of the homologous E2 sequences. Gene expression from GAL UAS is induced by the presence of 25 galactose but strongly repressed by glucose. Thus, expression of the E2 sequences and E2 homologous sequences may be regulated by choice of yeast culture medium. In addition, plasmid pYE2 contains a ura gene, a selectable marker that permits growth of hosts on 30 selective media lacking uracil.

Figure 1 depicts the sequence of amino acids 285-410 of the wild type BPV1 E2 protein (E.Y. Chen et al., "The Primary Structure and Genetic Organization of the Bovine Papillomavirus Type 1 Genome", Nature, 35 299, 529-34 (1982)). This region of the BPV1 E2

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prot in is known as th DNA binding domain (Giri and Yaniv, supra). Figure 1 als depicts chang s in the amino acid s quence of th BPV1 E2 DNA binding domain which characterized various E2 transcription repressor 5 mutants prepared according to this invention. As shown in Figure 1, mutations were scattered throughout the DNA binding domain. Several of the dimerization defective E2 mutants were characterized by two or three nucleotide alterations from the native E2 protein 10 sequence.

The cysteine at position 340 of the wild type E2 protein in Figure 1 is present in all papillomavirus E2 proteins whose sequences are known. Furthermore, DNA binding activity of E2 protein is dependent on the 15 presence of reducing agents. Accordingly, we generated mutations at position 340 in order to determine the criticality of that cysteine. We used site-directed mutagenesis to substitute the three other nucleotid s for G and for C in the TGC codon for cysteine 340 in 20 the BPV1 E2 gene. We performed the site-directed mutagenesis according to the method of Kunkel et al., "Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection", Methods Enzymol. 154, pp. 367- 82 (1987).

25 Figure 2 identifies various E2 trans- activation repressor mutants prepared according to this invention and summarizes their DNA binding, dimerization and repression activities, as assayed in the following examples.

30

EXAMPLE 2

Screening Mutations By trans-activation Of A Reporter Gene In Yeast

We tested the E2 mutants for trans- activation of a reporter gene in an E2 trans-activation

reporter strain. More specifically, we used pYE2, carrying the population of E2 mutations, to transform reporter strain BGW1-7A, a yeast strain which contained yeast reporter plasmid pBY-4 (Morrissey et al., *supra*).
5 Plasmid pBY-4 contains a β -galactosidase reporter gene under the control of an E2-dependent promoter, plus a Leu2 gene, which serves as a selectable marker. Expression of the β -galactosidase gene in pBY-4 was rendered E2 dependent by having it under the control of
10 an appropriately placed cyc-1 minimal promoter preceded upstream by four E2 binding sites. We then selected for transformants that contained both plasmids on a 2% glucose/minimal medium without leucine and uracil. Transfer of cells to glucose minimal medium lacking
15 leucine and uracil provided strong positive selection pressure for transformants harboring both plasmid pYE2 and plasmid pBY-4, while leaving expression of E2 sequences uninduced.

Although not required, a step involving such
20 selection pressure without expression of E2 sequences, immediately following transformation, is preferred, in order to exclude the possibility that expression of E2 protein or E2 homologues might confer a selective disadvantage that would discriminate against the
25 desired transformants in a mixed population.

The colonies selected were then replicated onto selective yeast minimal medium containing 100 mM potassium phosphate (pH 6.9), 2% galactose, and 0.004% X-gal. X-gal is a colorless β -galactosidase
30 substrate (5-bromo-4-chloro-3-indolyl β -glucoside) that yields a blue product upon cleavage by β -galactosidase. Galactose induced expression of the E2 gene carried on pYE2 and X-gal gave a color indication of E2-dependent activity of the β -galactosidase gene carried on plasmid
35 pBY-4. On media containing galactose and X-gal,

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transformants expressing trans-activating E2 homologues were blue, while colonies expressing non-activating homologues were light blue or white. After incubating the cultures for 48 hours at 30°C, we visually assayed 5 colony color, on a scale of 1-8. Approximately 10-15% of the colonies were white or light blue. All E2 mutants listed in Figure 2 were originally isolated as white or light blue colonies. White colonies harbored trans-activation-abolishing mutations, while light blue 10 colonies harbored trans-activation-reducing mutations. Mutants 366Y/376L, 386W, 360S, 399I, 408*, 411*, and 3SLI were isolated as light blue colonies in the initial screening. The other mutants listed in Figure 2 were isolated as white colonies. Dark blue 15 colonies harbored either unmutated E2 sequences or E2 mutations that did not reduce E2 trans-activation, and thus were discarded.

The E2 plasmid, pYE2, was isolated from each mutant clone and the mutagenized E2 insert of each 20 clone was sequenced by standard methods.

As detailed above in Example 1, the five mutants at cys340 did not arise from our screen, but were generated by site-directed mutagenesis.

EXAMPLE 3

25 Expression Of E2 Protein And E2 Homologues In Yeast And In E.coli

We analyzed the selected light blue or white transformants for expression of full-length E2 proteins as follows. Since mutations resulting in premature 30 termination codons or unstable E2 proteins were not desired, we extracted total protein from cultures of the selected light blue and white colonies and tested that protein by standard immunoblot techniques. Only mutant clones that produced nearly wild-type levels of

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protein that reacted with E2 antibodies and that also had a molecular weight of ab ut 50 kD were further characterized.

First, we cultured each of the selected light
5 blue or white transformants in 50 ml of selective minimal medium containing 2% galactose, for 7 hrs at 30°C. To extract E2 proteins, we harvested the cells by centrifugation and washed them with protein extraction buffer (200 mM Tris/HCl (pH 8.0), 400 mM 10 ammonium sulfate, 10 mM magnesium chloride, 1 mM EDTA and 10% glycerol (v/v)). We then suspended the washed cells in 2 volumes of protein extraction buffer supplemented with 5 mM DTT and the following protease inhibitors: 1 mM PMSF, 1mM TLCK, pepstatin and 5mM 15 benzamidine hydrochloride.

After addition of washed 0.45 mm diameter glass beads (about equal in volume to the yeast cell pellet), we disrupted the yeast cells by vigorous vortexing 6 times, for 30 sec. each time. Heavy
20 insoluble debris was removed by a first round of centrifugation and the supernatant clarified by centrifugation at about 13,000 x g for 1 hr at 4°C. We added an equal volume of cold, saturated ammonium sulfate solution to the clarified supernatant and
25 allowed proteins to precipitate on ice for 15 min. The precipitated proteins were pelleted by centrifugation in a fixed angle JA-17 rotor at 13000 x g for 10 minutes at 4°C and then dissolved in 50 µl of solubilization buffer (25 mM Tris/HCl (pH 8.0), 2 mM 30 EDTA, 20% glycerol (v/v), 1 mM DTT and the same mixture of protease inhibitors used in the protein extraction buffer.

We expressed the C-terminal 126 amino acids (plus an N-terminal methionine residue) of the BPV1 E2
35 polypeptide and the corresponding mutant polypeptides

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(homologues) in the E.coli expression vector pET8C, as described by Studier, "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes", Methods Enzymol., 185, pp. 60-90 (1990), after creating a KpnI site immediately 3' to the NcoI site and ATG codon. Mutant E2 sequences were then transferred into pET8C-E2 as KpnI-BstX1 fragments. The pET8C-E2 expression vectors were induced to express the E2 homologues as described by Studier (supra), following transformation of the expression host E.coli strain BL21(DE3)pLYSS (Studier, supra). We produced 50 ml cultures of the transformed expression host strain grown under inducing conditions and harvested the cells by centrifugation.

The harvested cells were suspended in 4 ml of 20 mM MES (pH 6.0) containing 1 mM PMSF and subjected to freeze-thaw lysis. Insoluble debris was removed by centrifugation. The E2 polypeptides and E2 homologues were partially purified by chromatography on S-Sepharose (Pharmacia-LKB, Piscataway, NJ). We applied the protein solution to a 0.2 ml column of S-Sepharose that had been pre-equilibrated with the freeze-thaw lysis buffer. The column was washed with 20 mM MES (pH 6.0), 100 mM NaCl, 5 mM DTT and 1 mM EDTA. E2 polypeptides and E2 homologues were then eluted from the column with a solution containing 20 mM MES (pH 6.0), 600 mM NaCl, 5 mM DTT, 1 mM EDTA, 10% glycerol (v/v), 1 mM PMSF, 1 μM pepstatin, 2 μg/ml leupeptin and 2 μg/ml aprotinin. As described below, we then tested for the presence of E2 homologues in the eluate by conventional immunoblot procedures, which may be carried out by those of ordinary skill in the art using standard techniques.

We resolved proteins in the eluate according to molecular weight by SDS polyacrylamide gel electrophoresis. Following electrophoresis, we

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transferred the resolved prot ins onto nitrocellulose membranes by standard blotting techniques. We then treated the nitrocellulose membranes with bovin s rum albumin to saturate non-specific protein binding sit s
5 on the membrane and exposed the membrane to polyclonal rabbit anti-E2 serum at a serum dilution of 1:2500, for 2 hours, at room temperature. After washing the membrane to remove unbound antibodies, we visualized antibodies bound to electrophoretic protein bands via
10 alkaline phosphatase conjugated to antibodies that bind to rabbit immunoglobulins.

EXAMPLE 4

DNA Binding Assays

In order to determine which mutant E2
15 polypeptides (homologues) bound to E2 DNA binding sites, we carried out DNA binding assays.

We first mixed between about 0.5 and 4.0 μ l of partially purified E2 polypeptides or homologues, at a concentration of about 1 ng/ μ l (prepared as described
20 in Example 3 above) with about 1.5 μ g of poly dI-dC and about 300 ng of sheared salmon sperm DNA, in a total volume of about 20 μ l, for 10 min. at 4°C. We then added between about 0.5 and 2.0 ng of end-labelled DNA fragments (about 10,000 cpm/reaction) containing one,
25 two or four E2 DNA binding sites and placed the mixture on ice. The DNA fragments (probes) containing E2 binding sites consisted of NsiI restriction fragments from pBY-1 (one E2 binding site), pBY-2 (two E2 binding sites), or pBY-4 (Morrissey et al., supra). After 30
30 minutes, we added 1/10 volume of 20 mM Hepes (pH 7.5), 20% glycerol (v/v) and 0.25% bromophenol blue to the DNA-protein mixture, for electrophoresis. We then resolved DNA-protein complexes from unbound DNA and protein by electrophoresis in 4-5% polyacrylamide gels

for about 3-4 hours, at 150 v. The electrophoresis buffer was 0.5 x TBE. Following electrophoresis, gels were dried and exposed to X-ray film. Our DNA binding assay was in accord with well-known methods (see generally: F. Ausubel et al., "Mobility Shift DNA Binding Assay Using Gel Electrophoresis", in Current Protocols in Molecular Biology, pp. 12.2.1-12.2.10 (1988)).

Figure 3 shows autoradiograms of electrophoresis gels from these DNA binding "gel shift" assays. As depicted in that figure, the sample in each lane included radioactive DNA probe containing E2 binding sites. The DNA in panels A and B contained two E2 binding sites, and the DNA in panel C contained 4 DNA binding sites. Sample designations are as follows: "p", radioactive DNA probe containing E2 binding sites, in the absence of added protein; "wt", native E2 repressor; "333", mutant polypeptide 333V; "337", mutant polypeptide 337L; "339", mutant polypeptide 339M; "344", mutant polypeptide 344L; "360", mutant polypeptide 360S; "316", mutant polypeptide 316Y; "370", mutant polypeptide 370I; "340R", "340F", "340Y", "340S", "340G" and "3SLI" refer to mutant polypeptides having those designations. The basis of the assay is that protein bound to the DNA probe slows electrophoretic migration of the DNA. Thus, binding of protein to the DNA causes the DNA band to be "shifted" from its electrophoretic position observed in the absence of bound protein. Figure 3 shows that mutant polypeptides 333V, 337L, 339M, 344L, 340F, 340R, 340Y and 360S did not "shift" the electrophoretic position of the DNA probe, and thus they did not bind to the E2 binding sites on the DNA probe. Mutants 333V, 337L, 339M, 344L, 340R, 340Y, 340F, 403 all failed to stably bind the E2 DNA element in this gel-shift assay.

To further characterize homologues that were dimerization defective, we performed super-shift DNA binding assays, using monoclonal antibodies to BPV1 E2 protein. The super-shift assays were carried out to 5 determine whether dimerization defective E2 homologues would bind to E2 DNA binding sites when held together in pairs by monoclonal antibodies, to simulate dimerization. In the super-shift assays, we first incubated the E2 homologues on ice with between about 2 10 to 4 µl of culture medium (DulBecco's modified medium with 10% fetal calf serum) from a monoclonal antibody-producing hybridoma cell culture, for 30 min., before addition of labelled DNA. We then placed the mixture on ice. After 30 min., we added 1/10 volume of 15 20mM Hepes (pH 7.5), 20% glycerol (v/v) and 0.25% bromophenol blue to the DNA-protein-antibody mixture, for electrophoresis. Electrophoresis was as in the DNA binding assay described above.

Figure 4 shows autoradiograms of 20 electrophoresis gels from a super-shift DNA binding assay performed with dimerization-defective mutant polypeptide 360S. In that figure, Gel A shows that in the absence of anti-E2 monoclonal antibody, mutant polypeptide 360S did not bind to DNA probes having 1, 2 25 or 4 E2 binding sites. Gel B shows that in the presence of anti-E2 monoclonal antibody, however, mutant polypeptide 360S did exhibit binding to DNA probes having 2 or 4 E2 binding sites. Gel A samples were as follows: "1P", DNA probe with one E2 binding 30 site, in the absence of added protein; "1A", DNA probe with one E2 binding site, in the presence of mutant polypeptide 360S; "1B", DNA probe with one E2 binding site, in the presence of native E2 repressor; "2P", DNA probe with two E2 binding sites, in the absence of 35 added protein; "2A", DNA probe with two E2 binding

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sites, in the presence of mutant polyp ptid 360S; "2B", DNA probe with two E2 binding sites, in the presence of native E2 repressor; "4P", DNA probe with four E2 binding sites, in the absence of added protein;

5 "4A", DNA probe with four E2 binding sites, in the presence of mutant polypeptide 360S; "4B", DNA probe with four E2 binding sites, in the presence of native E2 repressor. Gel B samples were as follows: "1P", DNA probe with one E2 binding site, in the absence of

10 added protein; "1A", DNA probe with one E2 binding site, in the presence of mutant polypeptide 360S and monoclonal antibody; "1B", DNA probe with one E2 binding site, in the presence of native E2 repressor and monoclonal antibody; "2P", DNA probe with two E2

15 binding sites, in the absence of added protein; "2A", DNA probe with two E2 binding sites, in the presence of mutant polypeptide 360S and monoclonal antibody; "2B", DNA probe with two E2 binding sites, in the presence of native E2 repressor and monoclonal antibody; "4P", DNA

20 probe with four E2 binding sites, in the absence of added protein; "4A", DNA probe with four E2 binding sites, in the presence of mutant polypeptide 360S and monoclonal antibody; "4B", DNA probe with four E2 binding sites, in the presence of native E2 repressor

25 and monoclonal antibody.

In the initial screening of mutants for loss of E2 trans-activation, we noted that while mutant 360S was unable to activate the promoters with one or two E2 binding sites, trans-activation was approximately 40% of native E2 protein control levels in assays involving four E2 binding sites. In DNA binding assays with DNA probes having one or two E2 binding sites, mutant polypeptide 360S had practically no DNA binding activity, but in assays with a DNA probe having four E2 binding sites, 360S bound a small fraction --



approximately 1% of the DNA probe. This suggested that the 360S mutation might retain slight residual dimerization activity.

We complemented the dimerization defect of mutant polypeptide 360S with a monoclonal antibody, with the two antibody "arms" holding two 360S monomers in close proximity, to simulate dimerization. Monoclonal antibody (Mab) B202, whose epitope is immediately upstream from the DNA binding domain, or Mab B201, whose epitope maps further upstream between amino acids 160 and 220 of E2 protein, were included with the 360S polypeptide and DNA the probe. While monoclonal antibodies are preferred, polyclonal antibodies prepared by conventional techniques may also be employed in super-shift assays. The presence of Mab 202 restored practically complete binding of polypeptide 360S to DNA probes having two or four E2 binding sites. Mab 202 did not restore binding of 360S to the probe having only one E2 binding site (Figure 4). Mab 201 was only 5-10% as effective in restoring binding of mutant polypeptide 360S to E2 binding sites (data not shown). This was predictable, since the epitope of Mab 201 was further from the E2 DNA binding domain, which contains the E2 dimerization region, than was the Mab 202 epitope. We therefore expected Mab 201 to be less efficient at holding the 360S dimerization regions together than was Mab 202. To exclude the possibility that the monoclonal antibody binding restored DNA binding by altering the conformation of the 360S polypeptide, we performed super-shift assays on 360S with normal dimeric B202 antibodies, which bind two E2 polypeptides (or homologues), and monomeric B202 antibody fragments, which bind only a single E2 polypeptide, and therefore do not result in simulated E2 dimers. While the normal dimeric Mab 202 allowed 360S

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to bind to DNA, the monomeric form of Mab 202 did not rest re binding of 360S to DNA in gel shift DNA binding assays. Separate controls confirmed that the monomeric form of Mab 202 did bind to E2 polypeptides. These 5 data strongly support our belief that E2 monomers cannot bind to E2 DNA binding sites.

Since the E2 binding domain has no primary sequence homology to that of any other known transcription factor, the amino acids of E2 responsible 10 for DNA binding interactions ("DNA contact subdomain") were unknown prior to the instant invention.

Four of the mutant polypeptides of this invention, 333V, 337L, 339M and 344L, were isolated from a twelve amino acid span from positions 333 to 344 15 of E2 protein, which is a highly conserved region of that protein among all papillomaviruses. All were isolated as white colonies on the initial screen (Example 2). All failed to stably bind the E2 DNA element by gel-shift assay. Replacement of glycine 333 20 with valine also prohibited dimer formation (see Example 5), but since the other mutations (337L, 339M, 344L) existed as pre-formed dimers, we inferred that this region was responsible for DNA interactions. These latter three mutations altered the positively 25 charged amino acids glutamine, lysine and arginine, recognized to be involved in protein nucleic acid interactions. 333V also appeared to be transcriptionally distinct from other dimerization defective mutants (described below) which demonstrated activity with four E2 binding sites, while 333V did 30 not. The high conservation of glycine at this position suggested that it is critical for proper tertiary folding of the C-terminal portion of E2 protein.

Comparison of the amino acid sequence of the 35 region (amino acid residues 333 to 344) of the native

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E2 polypeptide to the DNA binding domain of other transcription factors failed to reveal similarities to the helix-turn-helix, helix-loop-helix, homeodomain, β -sheet, or zinc finger classes of DNA binding domains.

- 5 This region of the E2 polypeptide includes several basic amino acids and no acidic residues, yet bears virtually no primary sequence homology to the basic region of the jun/fos family of transcription factors, which has been shown to be required for their DNA
10 binding capability. In common with these, however, this E2 domain also contains a central cysteine (amino acid 340).

The 340R, 340Y and 340F E2 mutations, in which cysteine 340 was replaced with arginine, tyrosine or phenylalanine, respectively, had comparable characteristics to the mutations isolated in this region by chemical mutagenesis and phenotypic selection. These failed to trans-activate the E2 dependent promoter with one, two or four E2 elements, and were likewise defective for DNA binding by gel shift (Figure 3). These cysteine mutants were able to dimerize (see Example 5). These data suggest that substitution of bulky amino acids at cysteine 340 blocked DNA interactions, not through inhibition of protein-protein interactions, but through destabilization of the DNA contact subdomain.
20
25

EXAMPLE 5

In Vitro Dimerization Assay

- In order to determine which mutant E2 polypeptides retained the capacity to form dimers, subunits of dimeric E2 proteins were covalently bound by standard cross-linking reactions. Reaction conditions were adjusted so that the covalent bonding between subunits of pre-existing dimers occurred
30

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r adily, with minimal covalent bonding between monomers. Following the cross-linking reaction, the standard technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis ("SDS-PAGE"), which 5 separates proteins on the basis of size, was employed to determine which E2 mutations yielded polypeptides that formed dimers. Wild type E2 protein dimerizes in the absence of DNA.

For use in the cross-linking reactions, we 10 prepared crude extracts of E2 homologues from cultures of yeast clones (as described in Example 3, *supra*). We then carried out cross-linking by exposing samples from those yeast crude extracts to an ultraviolet (354 nm) light box, for 30 sec., at a distance of 1 cm.

15 Following the cross-linking reactions, we prepared the protein samples for SDS polyacrylamide gel electrophoresis by adding SDS to a final concentration of 3% and β -mercaptoethanol to a final concentration of 5%. We then heated the samples and maintained them at 20 65°C for about 3 min. We used a 9% polyacrylamide gel to resolve proteins. Following electrophoresis, we transferred the resolved proteins onto nitrocellulose sheets using standard electroblotting techniques. We then detected mutant E2 monomers and cross-linked 25 dimers (at about 50 kD and about 100 kD, respectively) by immunoblot using polyclonal antisera to BPV1 E2 protein.

The results of these assays are set forth in Figure 2. As shown in that figure, the dimerization-30 preventing mutations mapped from amino acid position 360 to at least amino acid position 402 of E2 protein, with the dimerization region potentially extending almost to the end of the polypeptide (i.e., position 410). Mutation 360S was the only single amino acid 35 change that removed all dimerization activity. Th

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360S mutation interestingly alt red a highly conserved tryptophan r sidue shar d among the papillomaviruses. Analyses by UV cross-linking of 360S utilizing yeast and bacterial expression vectors demonstrated the 5 inability of this tryptophan point mutant to form dimers. Mutant 360S was a very poor repressor, strongly supporting our belief that the dimerization function is required for repression of papillomavirus trans-activation and viral replication. Dimerization- 10 defective mutants, 3SLI and 402* likely had intact DNA binding domains, since like mutant 360S, 3SLI and (to a lesser extent) 402* could super-shift -- they could bind DNA and give a shift in a band retardation assay in the presence of a monoclonal antibody that 15 recognizes the DNA domain binding of E2 protein (see Example 4). We believe that this super-shift activity resulted from the E2 mutant polypeptides being held together as a simulated dimer by the antibody. Mutants such as 402*, which have small insertions or deletions, 20 may have failed to dimerize due to gross perturbations in protein folding. Thus, it is not clear whether the region of that mutation is directly involved in dimerization.

E2 Dimerization Function

25 We have partially characterized a previously published (Haugen et al., supra) E2 mutant, 3812i (called 402* herein), which is DNA binding-defectiv and inactive in our yeast trans-activation assay system, with even four E2 binding sites. Mutant 402* 30 has an in-frame insertion of 4 amino acids at position 402. Analysis of the biochemistry of this mutant revealed that it does not dimerize in vitro and does not bind to E2 binding sites on DNA in DNA binding (gel shift) assays. However, 402* can be complemented for

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DNA binding with a polyclonal anti-E2 serum in a super-shift assay. These results suggest that the E2 protein dimerization region itself spans, or is affected by, the region from amino acids 360 to 402 of E2 protein.

5 Other mutations in the E2 protein dimerization region were isolated as light blue colonies on our initial screening. Mutants 3SLI and 366Y/376L displayed intermediate levels of transcriptional activation, mutant 399I converted a C-
10 terminal methionine to isoleucine and this had slightly reduced ability to activate the E2 dependent promoters. Mutant 386W replaced a highly conserved arginine with tryptophan and was also found to be partially defective for promoter activation. Biochemical characterization
15 of these reduced activation mutants demonstrated DNA binding activity in gel shift assays (Example 4) and formed dimers *in vitro*. In the repression studies, mutant proteins 386W and 399I were efficient repressors (see Example 6). These mutant polypeptides dimerize
20 and bind DNA. Mutant protein 3SLI had a reduced level of E2 transcriptional repression. Biochemical studies demonstrated that it has decreased dimerization capability, but not as defective as the 360S mutant protein. Mutations 408* and 411* affected the 3'
25 terminus of E2. While this segment of E2 is not highly conserved among the papillomaviruses, the loss of dimerization activity upon insertion of four amino acids at position 402 revealed the requirement of this region for dimerization. Nonetheless, both 408*, which
30 had alteration of the last 3 amino acids and added an additional 8 residues, and 411*, which had the translational stop codon replaced with a leucine codon, resulting in an extra 22 C-terminal amino acid residues, retained trans-activation function in large
35 part. Consistent with its defective dimerization,

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mutant polypeptide 402* was not able to repress E2 trans-activation. Mutant polypeptides 408* and 411* both can bind DNA and dimerize as 126 amino acid forms purified from the E.coli expression host. 408* was a 5 weak repressor, but 411* failed to repress. It is not clear why 411* does not repress E2 trans-activation, but we believe that it may have reduced ability to form inactive heterodimers with full-length E2 proteins due to the 22 amino acid peptide fused to its carboxyl 10 terminus.

These genetic and biochemical analyses suggest that the region of E2 protein that interacts with DNA directly is between about amino acids 333 and 344, and that dimerization activity is encoded by a 15 complex domain that spans the segment between about amino acids 360 and 402. Accordingly, we believe that the DNA binding recognition and the dimerization functions of E2 proteins are separable and mediated by two novel motifs. A short basic region, unlikely to be 20 helical, is required for DNA binding but not dimerization. While a conserved central cysteine in this motif is not necessary, this represents a critical position for modifying the DNA binding capacity of E2 protein, since replacement of cysteine with large amino 25 acids adds efficiently abrogated DNA binding. The dimerization motif includes a critical tryptophan at position 360 in BPV-1 E2 polypeptide.

It should be noted that DNA binding capacity was lost by a mutant in every instance in which 30 dimerization capacity was lost. These results indicate that dimerization is a prerequisite for DNA binding. However, some mutant gene products that lost the capacity for DNA binding retained the capacity for dimerization. In those mutant gene products, which 35 represent the novel class of E2 trans-activation

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r pr ss rs f this invention, the dimerization function was separated from th DNA binding function. Mutants 337L, 339M, 340F, 340R, 340Y and 344L ar included in this group. These mutants that dimerized without 5 binding to E2 DNA binding sites were further tested for capacity to repress E2-dependent trans-activation in cultured animal cells.

EXAMPLE 6

Repression Of E2-Dependent trans-activation In Cultured Animal Cells

We next assayed the capacity of E2 mutants that dimerized without binding to E2 DNA binding sit s to repress E2-dependent trans-activation in cultured mammalian cells. In this assay, an E2 dependent 15 reporter plasmid (characterized by either the gene encoding chloramphenicol acetyltransferase (CAT) or the gene encoding human growth hormone (hGH) driven by a truncated SV40 promoter having three upstream E2 binding sites), the full-length wild type E2 trans- 20 activator protein driven by the actin promoter and the mutant clones in an E2-repressor format starting at nucleotide 3089 of BPV, (i.e., amino acid 160), also driven by the actin promoter, were simultaneously introduced into cultured mouse embryo fibroblast cells 25 by well-established electroporation techniques. The E2 repressor DNA was present at a four-fold excess. Th E2 repressor assay was performed at an E2 trans- activator level below the saturation level, sinc high amounts of E2 repress transcription, perhaps by 30 "squelching". Transfections which resulted in the greatest E2 inductions also gave the best E2 repression (see Table II, infra) and the greatest reproducibility.

The reporter plasmid was constructed so that expr ssion of the r porter gene was highly dependent on

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E2 trans-activation (i.e., it comprised one, two or four E2 DNA binding sites appropriately placed relative to the promoter and reporter gene coding sequence). The choice of a reporter gene is largely a matter of convenience. In general, any gene whose expression, either directly or indirectly, results in a product that can be measured with reasonable accuracy and reliability can be used as a reporter gene. Preferred reporter genes for the assay of E2 trans-activation in the cultured mammalian cells according to this invention are the gene encoding hGH and the gene encoding CAT.

We constructed the hGH reporter plasmid, pXB332hGH, in a two-step process (Figures 5 and 6). First, we constructed pXB332 by inserting the E2-dependent promoter (SalI-HindIII fragment) from the E2-dependent reporter plasmid pC515-9 (Hawley-Nelson et al., supra, (1988)) into plasmid vector pXB100 (see Figures 5 and 7) that had been previously cleaved with XbaI and HindIII, to form plasmid pXB332. We then inserted the hGH gene as a HindIII-EcoRI fragment from pOGH (Nichols Institute, San Juan Capistrano, California) into pXB332 that had been cleaved with HindIII and EcoRI to create pXB322hGH (Figure 6). We constructed the CAT reporter plasmid according to a published method (P. Hawley-Nelson, supra).

The E2 trans-activator plasmid vector comprised a full-length native BPV1 E2 gene from pCO-E2 (Hawley-Nelson et al., supra), operatively linked to control sequences that rendered its expression essentially constitutive. Thus, the E2 trans-activator plasmid directed synthesis of E2 protein for trans-activation of the reporter gene. In order to ensure that repressor effects were observable, however, the promoter controlling expression of the full-length E2

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gene was not as active as to yield saturating levels of full-length E2 protein in the transfected mammalian cells of the repressor assay system. If the E2 trans-activator gene is overexpressed, repression data are unreliable. In a preferred embodiment of this invention, a chicken β -actin promoter is employed for expression of the E2 trans-activator gene.

We expressed the native E2 coding sequences and mutant E2 coding sequences from the chicken β -actin promoter (T.A. Kost et al., "The Nucleotide Sequence of the Chick Cytoplasmic β -Actin Gene", Nucl. Acids Res., 11, pp. 8287-8301 (1983); A. Seiler-Tuyns et al., "Expression and Regulation of Chicken Actin Genes Introduced into Mouse Myogenic and Non-Myogenic Cells", Proc. Natl. Acad. Sci. USA, 81, pp. 2980-84 (1984)) in animal cells using vector pXB101 (See Figure 7).

We constructed plasmid pXB101 in a 2-step process (Figure 7). Two oligonucleotides were synthesized and annealed to form a polylinker:

20 5' CTCGAGAAGCTTGACGGATCCG 3' (SEQ ID NO:18)
 3' TGCAGAGCTCTCGAACTGCCTAGGCTTAA 5' (SEQ ID NO:19)

This polylinker contained XhoI, HindIII, and BamHI restriction sites internally, with an AatII compatible overhang at the 5' end and an EcoRI compatible end at the 3' end. We then cleaved plasmid pBG312 (R.L. Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance and Expression of the Human Gene in Animal Cells", Cell, 45, pp. 685-98 (1986)) with AatII and EcoRI to release a fragment containing the Ad-2 promoter of pBG312. We inserted the polylinker into the cleaved pBG312 in place of the Ad-2 promoter to form the promoterless vector pXB100. We then cleaved pXB100 with XhoI and BamHI (exploiting the polylinker sites) and inserted the chicken β -actin

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promoter from p8Act-1 (Kost et al., supra) as a 280 bp XhoI-BamHI fragm nt, to form pXB101.

For expression of native full-length E2 protein, we inserted a 1866 bp BamHI fragment from pCO-
5 E2 (Hawley-Nelson et al. supra) into the BamHI site of pXB101, to form plasmid pXB323 (Figure 8).

A polypeptide consisting of the C-terminal 249 amino acids of the native E2 protein lacks trans-activation capacity and can repress trans-activation by
10 native full-length E2 protein (P.F. Lambert et al., supra). For expression of the native BPV1 E2
repressor, the 1362 bp NcoI-BamHI fragment of pXB323
15 (encoding the C-terminal 249 amino acids of the BPV1 E2 protein and starting with a methionine at the NcoI site), was inserted into the BamHI site of pXB101, to
form pXB314 (Figure 9). Synthetic oligonucleotides
were also inserted in order to join the NcoI cohesive
end of the 1362 bp fragment to the BamHI cohesive end
of pXB101. Those synthetic oligonucleotides are shown
20 below:

5' GATCCTTGCCGCCAC 3' (SEQ ID NO:20)
3' GAAACGGCGGTGGTAC 5' (SEQ ID NO:21)

In order to test E2 homologues for their capacity to repress E2 trans-activation, mutant forms
25 of the E2 DNA binding domain (from clones selected in the phenotype screening described in Examples 2 and 3 above) were inserted as KpnI-BstX1 fragments into KpnI-BstX1-cleaved pXB314 (see Figure 10). In this way, the C-terminal 126 amino acids of the polypeptide
30 consisting of the C-terminal 249 amino acids of the native E2 polypeptide were replaced with the corresponding mutant sequences to form each of the mutant E2 repressor plasmids assayed, including pEC337L, pE339M, pEC340F, pEC340R, pEC340Y and pEC344L.

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All transfections and assays were performed at subsaturating levels of the E2 trans-activator. This was done by using the moderately weak actin promoter to drive expression of the protein. Unless 5 otherwise indicated, all steps were carried out at room temperature.

The transfections were carried out on the mouse embryo fibroblast cell line Balb/c 3T3, clone A31 (S.A. Aaronson and G.J. Todaro, "Development of 3T3-Like Lines from Balb/c Mouse Embryo Cultures: Transformation Susceptibility to SV40", J. Cell Physiol., 72, pp. 141-48 (1968)), obtained from the American Type Culture Collection (ATCC accession no. ATCC CCL163). The 3T3 cell culture medium was 15 DulBecco's minimal essential medium (Gibco, Grand Island, NY), with 10% donor calf serum (Hazelton, Lenexa, KS) and 4 mM glutamine (Whittaker, Walkersville, MD). We maintained the 3T3 cell cultures in an incubator at 37°C, in an atmosphere containing 20 5.5% CO₂. Cells were grown in 100 mm culture dishes (Corning, Corning, NY, cat. no. 25020). The cells were passaged by washing with phosphate-buffered saline solution (Gibco) and treatment with trypsin (Gibco), (to remove adhering cells from the culture vessels), 25 followed by addition of fresh culture medium and dilution of cultures into vessels containing fresh culture medium.

Transient electroporations were carried out to measure the repression activity of the mutants. We 30 employed a commercially available electroporation device (Gene Pulser™, BioRad, Richmond, CA) and used an electroporation technique similar to that of G. Chu et al., "Electroporation For the Efficient Transfection of Mammalian Cells With DNA", Nucl. Acids Res., 15, pp. 35 1311-26 (1987) to introduce plasmids into the 3T3

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c 11s. In each electroporation, we used a total of 400 µg of DNA. Of that 400 µg, 20 µg was reporter plasmid, 20 µg was trans-activator plasmid and 80 µg was repressor plasmid. The remainder of the 400 µg was
5 made up with herring sperm DNA (Boehringer Mannheim,
Indianapolis, IN), that had been sonicated to fragments
of about 300 to 2000 bp in size. To a solution of the
DNA (0.4 ml) we added NaCl to a final concentration of
0.1 M and then we precipitated the DNA with 2.5 volumes
10 of ethanol. We pelleted the precipitated DNA in an
Eppendorf centrifuge, air-dried it in a tissue culture
hood and resuspended the DNA in 0.8 ml of 20 mM HEPES
(pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄ and
6 mM dextrose, ("1 x HeBS"). We allowed the DNA to
15 resuspend in the 1 x HeBS from about 3 to 24 hours.

For each electroporation, we removed about
5 x 10⁶ 3T3 cells (that had been passaged or fed on the
previous day) from a culture vessel by trypsin
treatment and pelleted the cells by centrifugation at
20 1000 rpm in a Damon/IEC HN-SII rotor (about 250 x g)
for 4 min. After removal of the medium above the
pelleted cells by aspiration, we resuspended the cells
in the DNA plus 1 x HeBS (see above). We then
transferred the solution containing the DNA and cells
25 to an electroporation cuvette. We immediately
discharged a 960 µFD capacitor, to yield about 240 v
for about 10 msec. We left the cells in the cuvette
for about 8 min. and then transferred them to a test
tube containing 10 ml of culture medium and pelleted as
30 above. We then aspirated the medium, resuspended the
cells in 10 ml culture medium, seeded them into a 10 cm
plate and returned the plate to the cell culture
incubator.

When using the hGH reporter gene, we
35 harvested the culture medium to assay for secreted hGH

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after 48 to 72 hrs. Alternatively, when using the CAT reporter, we harvest the electroporated cells after 48 to 72 hours. We controlled for cell number by counting cells, if using the hGH assay, and by measuring total protein concentration in the extracts, if using the CAT assay.

In order to quantitate expression of the reporter gene, we performed hGH assays according to the method of Selden, Protocols in Molecular Biology, 10 Greene Publishing Associates, New York, pp. 9.7.1 - 9.7.2 (1987). For hGH assays, we used a commercially available kit (Allegro™ Human Growth Hormone transient gene expression system kit, Nichols Institute, San Juan Capistrano, CA). We performed CAT assays according to 15 the method of C.M. Gorman et al. "Recombinant Genomes Which Express Chloramphenicol Acetyltransferase in Mammalian Cells", Mol. Cell Biol., 2, pp. 1044-51 (1982)). Positive and negative controls were employed, as appropriate. Such controls included transfection of 20 a reporter plasmid in the absence of a trans-activator plasmid (reporter background), transfection of a reporter plasmid in the presence of a trans-activator plasmid and absence of trans-activation repressor plasmid (unrepressed trans-activation) and transfection 25 of a reporter plasmid in the presence of a trans-activator plasmid and a plasmid for expression of the BPV1 E2 native repressor (i.e., the C-terminal 249 amino acids of the native E2 polypeptide).

In evaluating the E2 homologues, we utilized 30 data on reporter gene background level, E2 induction level and repression produced by the BPV1 E2 native repressor. Reporter gene background activity was calculated as reporter activity in the absence of E2 trans-activator protein. E2 induction level was 35 calculated as reporter activity in the presence of E2

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trans-activator protein divided by reporter background activity. Repression was calculated according to the following formula:

$$5 \quad \frac{[(ACT \text{ with E2}) - BKG] - [(ACT \text{ with E2 and REP}) - BKG]}{(ACT \text{ with E2}) - BKG}$$

Wherein:

ACT is activity of reporter

BKG is background activity of reporter

REP is repressor.

10 Table I (below) provides an example of results calculated with the above formula.*

TABLE I

	<u>Transfection</u>	<u>hGH (μg/ml)</u>	<u>Induction</u>	<u>Repression</u>
15	pXB332hGH (reporter)	0.1	---	---
20	pXB322hGH + pXB323 (reporter & <u>trans-activator</u>)	10.0	100-fold	---
25	pXB332hGH + pXB323 + pXB314 (reporter & <u>trans-activator &</u> <u>native repressor</u>)	1.0	10-fold	90.9%
30	pXB332hGH + pXB323 + pXB314.360S (reporter & <u>trans-activator &</u> <u>homologous repressor</u>)	7.0	70-fold	30.3%

* In this example, repression by the homologous repressor (360S) could also be expressed as 33% of the repression exhibited by the native repressor.

35 Table I illustrates a convenient and valid means of comparing repressor activities, with appropriate controls taken into account.

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Tabl II shows raw data and calculated values for several E2 repr ssor assays carried out as described above. In Table II, CPM represents sample radioactivity counts per minute; CPM - BKGD represents sample counts per minute minus background counts per minute; % Repression represents the value calculated for the mutant, from the formula above, multiplied by 100; % of Native Repression represents the % Repression value for the mutant divided by the repression value for the native repressor, calculated from the formula above; C represents the hGH or CAT reporter plasmid; 323 represents the trans-activator plasmid, pXB323; 314 represents the native repressor plasmid, pXB314; and the numbers followed by an upper case letter or an asterisk refer to the mutant polypeptide sequence being tested (see Figure 2).

TABLE II
E2 REPRESSION ASSAYS

	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM - BKGD</u>	<u>% REPRESSION</u>	<u>% OF NATIVE REPRESSION</u>
<u>CAT ASSAY #3</u>					
	C	234	---	---	---
	C + 323	3,278	3,044	---	---
	C + 314 + 323	378	144	95.3	---
25	C + 340S + 323	572.5	338.5	88.9	93.3

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	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM - BKGD</u>	<u>% REPRESSION</u>	<u>% OF NATIVE REPRESSION</u>
<u>CAT ASSAY #4</u>					
	C	161.5	---	---	---
5	C + 323	9,751	9,589.5	---	---
	C + 314 + 323	509.5	348	96.4	---
	C + 337L + 323	969.5	808	91.6	95.0
	C + 340R + 323	538	376.5	96.1	99.7
	C + 360S + 323	9,096	8,934.5	6.8	7.1
10	C + 402* + 323	5,868.5	5,707	40.5	42.0
<u>CAT ASSAY #5</u>					
	C	292	---	---	---
	C + 323	5,914	5,622	---	---
	C + 314 + 323	398	106	98.1	---
15	C + 339M + 323	1,738	1,446	74.3	75.7
	C + 344L + 323	579	287	94.9	96.7
	C + 360S + 323	2,656	2,364	57.9	59.1
	C + 402* + 323	3,102	2,810	50.0	51.0

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	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM - BKGD</u>	<u>% REPRESSION</u>	<u>% OF NATIVE REPRESSION</u>
<u>CAT ASSAY #6</u>					
	C	122.5	---	---	---
5	C + 323	2,867	2,744.5	---	---
	C + 314 + 323	218	95.5	96.5	---
	C + 337L + 323	418.5	296	89.2	92.4
	C + 339M + 323	725.5	603	78.0	80.9
	C + 370I + 323	313.5	191	93.0	96.4
10	C + 3SLI + 323	1,800	1,677.5	38.9	40.3
	C + 399I + 323	456	333.5	87.8	91.0
	C + 366Y/ + 323 376L	2,705.5	2,583	5.9	6.1
<u>CAT ASSAY #EP6</u>					
15	C	377	---	---	---
	C + 323	3,906	3,529	---	---
	C + 314 + 323	1,053	676	80.8	---
	C + 316Y + 323	3,607	3,230	8.5	10.5
	C + 340Y + 323	756	379	89.3	110.5
20	C + 344L + 323	1,429	1,052	70.2	86.9
	C + 370I + 323	3,214	2,837	19.6	24.3
	C + 3SLI + 323	2,524	2,147	39.2	48.5

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	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM - BKGD</u>	<u>% REPRESSION</u>	<u>% OF NATIVE REPRESSION</u>
<u>hGH ASSAY #2</u>					
	C	160	---	---	---
5	C + 323	9,937	9,777	---	---
	C + 314 + 323	415	255	97.4	---
	C + 337L + 323	862	702	92.8	95.3
	C + 340R + 323	452	292	97.0	99.6
	C + 344L + 323	1,680	1,520	84.4	86.7
10	C + 360S + 323	7,925	7,765	20.6	21.1
	C + 370I + 323	8,175	8,015	18.0	18.5
<u>hGH ASSAY #3</u>					
	C	303	---	---	---
	C + 323	14,522	14,218	---	---
15	C + 314 + 323	2,237	1,934	86.4	---
	C + 317STOP + 323	12,830	12,527	11.9	13.8
	C + 333V + 323	19,853	19,550	0	0
	C + 339M + 323	16,891	16,588	0	0
	C + 340F + 323	2,629	2,326	83.6	96.8

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	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM - BKGD</u>	<u>% REPRESSION</u>	<u>% OF NATIVE REPRESSION</u>
<u>hGH ASSAY #5</u>					
	C	239	---	---	---
5	C + 323	2,455	2,216	---	---
	C + 314 + 323	473	234	89.4	---
	C + 316Y + 323	2,287	2,048	7.6	8.5
	C + 333V + 323	4,275	4,036	0	0
	C + 340G + 323	486	247	88.8	99.3
10	C + 408* + 323	1,219	980	55.8	62.4
	C + 411* + 323	4,756	4,517	0	0
<u>hGH ASSAY #6</u>					
	C	169	---	---	---
	C + 323	5,207	5,038	---	---
15	C + 314 + 323	282	113	97.8	---
	C + 340Y + 323	289	120	97.6	99.8
	C + 386W + 323	1,147	978	80.6	82.4
	C + 408* + 323	2,024	1,855	63.2	64.6
<u>hGH ASSAY #7</u>					
20	C	110	---	---	---
	C + 323	1,692	1,582	---	---
	C + 370I + 323	335	225	85.8	?

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<u>SAMPLE</u>	<u>CPM</u>	<u>CPM - BKGD</u>	<u>% REPRESSION</u>	<u>% OF NATIVE REPRESSION</u>
<u>HIGH ASSAY # EP5</u>				
C	210	---	---	---
5 C + 323	11,207	10,987	---	---
C + 314 + 323	782	572	94.8	---
C + 317STOP + 323	10,943	10,733	2.4	2.5
C + 339M + 323	2,376	2,166	80.3	84.7
C + 340F + 323	1,475	1,265	88.5	93.4
10 C + 340G + 323	807	597	94.6	99.7
C + 340R + 323	763	553	95.0	100.2
C + 340S + 323	1,290	1,080	90.2	95.1
C + 366Y/ + 323 376L	8,314	8,104	26.3	27.7
15 C + 386W + 323	2,151	1,94	182.3	86.9
C + 399I + 323	1,750	1,540	86.0	90.7
C + 411L + 323	10,206	9,996	9.1	9.6

- Ratio of Repressor to trans-activator = 4:1

20 - Dashes indicate not applicable.

- Question mark indicates that % of Native Repression could not be calculated, because no control with the native repressor plasmid (C + 314 + 323) was done in that assay.

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Repression by a four-fold excess (by weight) of the native repressor was never below 80% in any assay. Reproducibility of the assay results shown in Table II was generally high. Mutant polypeptide 339M 5 did not repress at all in one assay but gave good repression in three other assays, when a different DNA preparation was used.

We tested each E2 homologue between two and four times for its ability to repress E2-dependent 10 trans-activation in mammalian cells. A compilation of trans-activation repression assay results is shown in Table III below. The repression activity is also summarized in Figure 2. It is clear from these assays that the C-terminal portion of E2 protein need not be 15 able to bind DNA in order to repress.

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TABLE III

Summary Of Mutant E2 Repressor Activity¹

<u>5</u>	<u>Mutant</u>	<u>Repression as % Decrease in Trans-Activation By E2</u>	<u>Repression As % of Repression By WT Repressor²</u>	<u>+/-</u>
	316Y	8.0	9.5	-
	317STOP	7.1	8.1	-
	333V	0 ³	-	-
	337L	90.4	93.7	+
10	339M	76.2	78.3	+
	340F	86.1	95.1	+
	340G	91.7	99.5	+
	340R	95.6	99.9	+
	340S	89.6	94.2	+
15	340Y	93.5	105.1	+
	344L	82.6	91.8	+
	360S	32.3	33.1	-
	366Y/376L	16.1	16.9	-
	370I	66.1	70.9	(±)
20	374S/375L/ 391I (3SLI)	39.0	44.4	-
	386W	81.4	84.6	+
	399I	86.9	90.8	+
	402*	45.2	46.6	-
25	(4 AA insert)			
	408*	59.5	63.5	(±)
	(11 AA insert)			
	411*	4.5	4.8	-

30 ¹ All values represent the average of two to four assays.

2 ² Each mutant repressor was compared to the native repressor in the same assay.

3 ³ "0" indicates that activation was slightly greater
35 in the presence of this mutant than in the control having no repressor present.

Table III shows a compilation of the results of all mutants which have been tested for repression. We arbitrarily defined a repressor as a protein which
40 repressed by at least 70% at a four-fold excess.

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Mutants 337L, 340F, 340R, 340Y and 344L, all of which could dimerize but did not bind DNA, repressed essentially as well as the native repressor.

Dimerization-defective mutants 360S, 3SLI and 402* did not repress. Mutants 316Y, 411*, and 366Y/376L did not repress, despite the fact that they were capable of forming dimers *in vitro*. However, mutant polypeptide 316Y appeared to be very unstable, suggesting that mutant polypeptides 316Y, 411* and 366Y/376L may have failed to repress as a result of their presence in the cells at very low concentrations, due to instability.

Thus, it appears that DNA binding is not necessary for repression of E2 *trans*-activation. Instead, a mechanism other than competition for DNA binding sites operates. Mutants which cannot dimerize -- or which do so very weakly -- 317STOP, 333V, 360S, 3SLI and 402* (4 AA insert) either repress poorly or not at all. We believe that the repressors of this invention act through dimer formation. More specifically, we believe that the repressors of this invention form heterodimers with the full-length E2 protein and thereby sequester it in an inactive form.

EXAMPLE 7

Repression of HPV E2-Dependent trans-Activation in Cultured Animal Cells

There is a high level of homology between the E2 binding domains of BPV1 and human papillomaviruses ("HPV"). Accordingly, we tested the ability of homologous HPV E2 sequences to function as E2 *trans*-activation repressors in a manner similar to that observed with the BPV E2 repressors described in Example 6, *supra*. To do this, we constructed vectors to express full-length native (*trans*-activating) HPV E2 protein and putative HPV E2 repressors.

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For expression of a full-length HPV E2 gene, we constructed plasmid pAHE2 (Fig. 11). Plasmid pAHE2 contains the E2 gene from HPV strain 16 operatively linked to the adenovirus major late promoter augmented by the SV40 enhancer upstream of the promoter. We isolated the HPV E2 gene from plasmid pHV16 (the full-length HPV16 genome cloned into pBR322), described in M. Durst et al., "A Papillomavirus DNA From Cervical Carcinoma And Its Prevalence In Cancer Biopsy Samples From Different Geographic Regions", Proc. Natl. Acad. Sci. USA, 80, pp. 3812-15 (1983) as a Tth111I-AseI fragment (Tth111I cleaves at nucleotide 2711, and AseI cleaves at nucleotide 3929 in the HPV16 genome). We blunted the ends of the Tth111I-AseI fragment in a DNA polymerase I Klenow reaction and ligated BamHI linkers (New England Biolabs, cat# no. 1021). We inserted this linker-bearing fragment into BamHI-cleaved plasmid pBG331, to create plasmid pAHE2.

Plasmid pBG331 is the same as pBG312 (Example 20; Fig. 7) except that it lacks the BamHI site downstream of the SV40 polyadenylation signal, making the BamHI site between the promoter and the SV40 intron unique. We removed the unwanted BamHI site by partial BamHI digestion of pBG312, gel purification of the 25 linearized plasmid, blunt end formation by DNA polymerase I Klenow treatment, self-ligation and screening for plasmids with the desired deletion of the BamHI site (Fig. 11).

To provide a positive control (i.e., an E2 repressor) for comparisons in the HPV E2 repression assays, we cloned the DNA sequence ("E2R fragment") encoding the 249-amino acid BPV E2 repressor into plasmid pBG331, to create pBG331E2R. To construct pBG331E2R, we removed the E2R fragment from pXB323 (Example 6; Fig. 8) by BamHI digestion, and then

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inserted that fragment into BamHI-cleaved pBG331. We used the same procedure to construct plasmid pBG331E2RN, a negative control, to express the dimerization-deficient BPV E2 sequence designated 360N.

- 5 Since it expresses a dimerization-deficient E2 polypeptide which does not repress E2 trans-activation, we incorporated pBG331E2RN into our repression assays to serve as a negative control. Plasmid pBG331 E2RN expresses a BPV1 E2 repressor with a tryptophan to 10 asparagine mutation at amino acid residue 360. This analogue is dimerization defective. It is similar to analogue 360S, described in Example 5, supra, but exhibits lower dimerization activity.

Based on our comparison of the BPV1 and HPV16 sequences, we expected the C-terminal 83 amino acid residues of the HPV E2 protein to exhibit dimerization and DNA binding activity, and thus to repress E2 trans-activation. We therefore constructed plasmid pHE2-85 from expression plasmid pBG331 (Figs. 12 and 13) by 15 inserting a 260 base pair NcoI-BamHI fragment ("E2-85") containing methionine and alanine codons immediately followed by codons for the C-terminal 83 amino acids of HPV16 E2. Similarly, we constructed plasmid pHE2-123 (Figs. 14 and 15) by inserting into pBG331 a 374 base 20 pair fragment ("E2-123") containing methionine and valine codons immediately followed by codons for the C-terminal 121 amino acids of HPV16 E2.

For construction of plasmids pHE2-85 and pHE2-123, we produced the necessary DNA fragments by 25 standard polymerase chain reaction ("PCR") techniques with pHV16 as the template. PCR chemicals and equipment are commercially available. For a general discussion of PCR techniques, see Chapter 14 of Sambrook et al., Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press (1989). The 30

nucleotide sequence of EA57, the PCR oligonucleotide primer for the 5' end of the 260 base pair E2-85 fragment, is set forth in the Sequence Listing under SEQ ID NO:28. The nucleotide sequence of EA52, the PCR 5 oligonucleotide primer for the 5' end of the 374 base pair E2-123 fragment, is set forth in the Sequence Listing under SEQ ID NO:29. The nucleotide sequence of EA54, the PCR oligonucleotide primer used for the 3' end of both fragments, is set forth in the Sequence 10 Listing under SEQ ID NO:30. We digested the PCR products with NcoI and BamHI and cloned the resulting fragments into NcoI/BamHI-digested expression plasmid pET8c (Studier et al, supra), to create plasmids pET8c-85 and pET8c-123. As depicted in Figures 13 and 15, we 15 cleaved pET8c-85 and pET8c-123 with NcoI and BamHI and transferred the fragments E2-85 and E2-123 into the unique BamHI site of plasmid pBG331, with the use of the BamHI-NcoI linker described in Example 6 (SEQ ID NO: 20 and SEQ ID NO: 21), to create plasmids pHE2-85 20 and pHE2-123.

We performed repression assays by transient transfections of mouse fibroblast 3T3 cells, using reporter plasmid pXB332hGH, as described in Example 6, supra. The HPV E2 repression assay results are shown 25 in Table IV, below. In Table IV, C represents the reporter plasmid, pXB332hGH. The other plasmids co-transfected in the repression assays have the same designation in Table IV as in the foregoing discussion. CPM represents sample radioactivity counts per minute; 30 CPM - BKGD represents sample counts per minute minus background (reporter plasmid alone) counts per minute; INDUCTION represents counts per minute for the combination of reporter plasmid with the trans- activator plasmid (pAHE2), divided by counts per minute 35 for the reporter plasmid alone; % REPRESSION represents

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the value calculated from the formula in Example 6,
multiplied by 100.

TABLE IV

HPV E2 REPRESSION ASSAYS

5 bGH assay HE2.4

	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM-BKGD</u>	<u>INDUCTION</u>	<u>% REPRESSION</u>
	C	461	---	---	---
	C + pAHE2	3,726	3,265	8.1	---
	C + pAHE2 + pBG331E2R	433	0	0	100.0
10	C + pAHE2 + pBG331E2RN	3,815	3,354	8.3	0
	C + pAHE2 + pHE2-85	879	418	1.9	87.2
	C + pAHE2 + pHE2-123	422	0	0	100.0

bGH assay HE2.5

	<u>SAMPLE</u>	<u>CPM</u>	<u>CPM-BKGD</u>	<u>INDUCTION</u>	<u>% REPRESSION</u>
15	C	513	---	---	---
	C + pAHE2	2,760	2,247	5.4	---
	C + pAHE2 + pBG331E2R	549	36	1.1	98.4
	C + pAHE2 + pBG331E2RN	2,405	1,892	4.7	15.8
	C + pAHE2 + pHE2-85	1,016	503	2.0	77.6
20	C + pAHE2 + pHE2-123	488	0	0	100.0

- Ratio of Repressor to trans-activator = 4:1

- Dashes indicate not applicable.

We observed a 5 to 8-fold level of trans-activation by full-length HPV16 E2 protein. This was approximately 10-fold lower than the level of trans-activation shown by full-length BPV1 E2 protein. To

compensate for the lower 1 v 1 of trans-activation, we used expression plasmid pBG331 for the HPV repression assay plasmid constructs. Plasmid pBG331 has a stronger promoter and therefore presumably expresses 5 higher levels of E2 protein than pXB101, which we used for plasmid constructs in the earlier BPV E2 repression assays described in Example 6.

As expected, the 249 amino acid BPV1 E2 repressor (positive control) completely repressed 10 trans-activation by the full-length HPV16 E2 protein, and the dimerization-defective BPV1E2RN analogue (negative control) showed negligible repression. Also as expected, the C-terminal 85 and 123 amino acids of HPV16 E2 protein functioned as repressors. The 85 15 amino acid HPV16 E2 protein fragment gave 87.2 and 77.6% repression, while the 123 amino acid HPV16 E2 protein fragment gave 100% repression in both assays. We believe that these HPV E2 repressors would maintain their repression activity upon introduction of 20 mutations that destroy DNA binding without inhibiting dimerization. Examples of such mutations are cysteine to arginine at position 300 in HPV16 E2 (which corresponds to position 340 in BPV1 E2), and arginine to leucine at position 304 (which corresponds to 25 position 344 in BPV1 E2).

EXAMPLE 8

Cellular Uptake of E2 Repressor Proteins

The E2 repressor proteins are not taken into cells at detectable levels. Thus, to deliver an E2 30 repressor protein into cells, we fused it to tat, a protein which naturally enters cells. Tat is a small, basic protein encoded by human immunodeficiency virus type I ("HIV-I"). It binds to the cell surface and gains entry by non-specific endocytosis in all cell

types so far tested. Uptake of more than 10 million tat molecules per cell has been observed. E2 repressors can be linked to tat by expression of a recombinant fusion protein in transformed host cells or 5 by chemical cross-linking of the two proteins. We have produced a tat-E2 repressor fusion protein in E. coli by recombinant DNA techniques.

From plasmid pXB314 (Example 6; Fig. 9) we isolated the NcoI-SpeI DNA fragment encoding the 249 10 amino acid BPV1 E2 repressor, E2R. (NcoI cleaves at nucleotide 296, and SpeI cleaves at nucleotide 1118 of pXB314.) We blunted the ends of this fragment by DNA polymerase I Klenow treatment and added the BglII linker (New England Biolabs, cat. no. 1090) described 15 in Example 6 (*supra*) and Fig. 9 (SEQ ID NO: 20 and SEQ ID NO: 21). We inserted this linker-bearing fragment into BamHI-cleaved (complete digestion) plasmid pTAT72. Plasmid pTAT72 is described in A. D. Frankel and 20 C. O. Pabo, "Cellular Uptake Of The Tat Protein From Human Immunodeficiency Virus", Cell, 55, pp. 1189-94 (1988). In plasmid pTAT72 there is a BamHI cleavage site within the tat coding region, near its 3' end, and a second BamHI cleavage site slightly downstream of the tat gene. The BglII linker joined the tat and E2 25 coding sequences in frame to encode a fusion of the first 62 amino acids of tat protein followed by a serine residue and the last 249 amino acids of BPV1 E2 protein. We designated this bacterial expression plasmid pFTE501 (Fig. 16). We expressed the tat-E2R 30 fusion protein in E. coli strain BL21 (DE3) as described in Studier et al., (*supra*). We purified the tat-E2R fusion protein from the insoluble fraction of E. coli according to the following procedure.

We pelleted the bacteria and resuspended them 35 in ten packed cell volumes of 25 mM Tris-HCl (pH 7.5),

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1 mM EDTA, 10 mM DTT, 1 mM PMSF, and lysed with 2 passages through a French press. We then pelleted the membrane fraction by centrifugation at 10,000 rpm for 30 minutes in a Sorval SS-34 rotor, and then 5 resuspended the membrane fraction in 6 M urea. We added solid guanidine-HCl to a final concentration of 6 M, and DTT to a concentration of 10 mM. After 30 minutes at 37°C, we clarified the solution by centrifugation at 10,000 rpm for 30 min. in a Sorval 10 SS-34 rotor. We then loaded the sample onto an A.5 agarose gel filtration column in 6 M guanidine-HCl, 50 mM sodium phosphate (pH 5.4), 10 mM DTT and collected tat-E2R-containing fractions from the gel filtration column, according to the appearance of a band of the 15 appropriate molecular size on Coomassie-stained SDS polyacrylamide electrophoresis gels. We loaded the gel filtration-purified sample onto a C₁₈ reverse phase HPLC column and eluted with a gradient of 0-75% acetonitrile in 0.1% trifluoroacetic acid. We collected the tat- 20 E2R fusion protein in a single peak with an apparent molecular weight of 40,000 Da.

We assayed cellular uptake of the tat-E2R fusion protein by indirect immunofluorescence in mouse fibroblast 3T3 cells. In the indirect 25 immunofluorescence procedure our primary antibody was either a rabbit polyclonal antibody against BPV1 E2, generated by injection of the purified C-terminal 85 amino acids of E2, or a rabbit polyclonal antibody against tat protein, generated by injection of the 30 purified 72 amino acid tat protein. We purified each of these 2 types of antibodies on an affinity column bearing the protein antigen against which the antibody was raised. Our secondary antibody was a rhodamine-conjugated goat anti-rabbit IgG (Cappel cat. no. 2212- 35 0081).

W seeded the 3T3 cells into 4-chamber tissue culture chamber/slides (commercially available from LabTek). The following day w added tat-E2R fusion protein or unfused tat protein to the culture medium at 5 1 mg/ml, with 0.1 mM chloroquine to inhibit lysosomal protease activity. Six hours later we observed immunofluorescence according to the following procedure.

We removed the culture medium and washed the 10 cells twice with phosphate buffered saline ("PBS"). We fixed the cells by treatment with 3.5% formaldehyde at room temperature, and permeabilized the cells by treatment with a solution of 0.2% Triton X-100, 2% bovine serum albumin ("BSA") in PBS containing 1 mM 15 $MgCl_2$ and 0.1 mM $CaCl_2$, which solution is designated PBS+. We blocked the cells by treatment with whole goat serum (Cappel cat. no. 5006-1380) diluted 1:30 with PBS+ containing 2% BSA, for 1 hour at 4°C. We added primary antibody at a 1:100 dilution in PBS+ 20 containing 2% BSA for 1 hour at 4°C, and then we added secondary antibody at a 1:100 dilution in 0.2% Tween-20, 2% BSA, in PBS+ for 30 minutes at 4°C. We washed the slides with 0.2% Tween-20, 2% BSA in PBS+, and then mounted in 50% glycerol in PBS. For viewing slide 25 preparations, we used a fluorescent microscope with a rhodamine filter.

With the tat antibody in the above procedure, we observed intense internal fluorescence in cells exposed to tat-E2R protein, and in positive control 30 cells exposed to non-fused tat protein. The E2 antibody gave intracellular fluorescence in cells exposed to the tat-E2R fusion protein, but not in cells exposed to unfused tat protein. No internal fluorescence appeared in negative controls that were 35 not exposed to the tat-E2R fusion protein or the

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unfused tat protein. In additional experimental controls, neither antibody resulted in significant intracellular fluorescence in cells to which an E2 repressor alone was added. The intensity and 5 subcellular location of fluorescence was similar whether the tat protein or the tat-E2R fusion protein was added to the cells. This indicates that the tat-E2R fusion protein entered the cells as efficiently as tat protein. These results indicate that the 10 antibodies were specific for their respective proteins, and that the tat protein can deliver the E2R protein into animal cells. An indication of the efficiency of the tat-induced uptake is that the intracellular fluorescence was far more intense when tat-E2R was 15 added to cells than when the tat-E2R or E2R gene constructs were expressed in transfected cells. We have obtained similar results in tests with other forms of tat and shorter forms of E2 repressors.

Microorganisms and recombinant DNA molecules 20 prepared by the processes of this invention are exemplified by cultures deposited in the In Vitro International, Inc. culture collection ("IVI"), in Linthicum, Maryland on January 18, 1991 and identified as:

25	314:	<u>E. coli</u> DH5/pXB314
	337L:	<u>E. coli</u> DH5/pEC337L
	339M:	<u>E. coli</u> DH5/pEC339M
	340F:	<u>E. coli</u> DH5/pEC340F
	340R:	<u>E. coli</u> DH5/pEC340R
30	340Y:	<u>E. coli</u> DH5/pEC340Y
	344L:	<u>E. coli</u> DH5/pEC344L.

These cultures were assigned accession numbers IVI 10262, IVI 10263, IVI 10264, IVI 10265, IVI 10266, IVI 10267 and IVI 10268, respectively. These cultures 35 were subsequently transferred from IVI to the American Type Culture Collection ("ATCC") in Rockville, Maryland, on June 20, 1991. They were assigned ATCC

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accession numbers 68740, 68735, 68736, 68737, 68738, 68739 and 68740, respectively.

Microorganisms and recombinant DNA molecules prepared by the processes of this invention are further 5 exemplified by cultures deposited in the American Type Culture Collection, Rockville, Maryland on January 24, 1992 and identified as:

pHE2-123

pHE2-85

10 BL21(DE3)/pLYSS/pFTE501

These cultures were assigned ATCC numbers 68896, 68897 and 68898, respectively.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that 15 our basic constructions may be altered to provide other embodiments which utilize the processes and products of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto, rather than by the specific 20 embodiments which have been presented hereinbefore by way of example.

In the following "Sequence Listing", we have provided nucleotide sequence and amino acid sequence information for the SEQ ID Numbers referred to in this 25 application. It should be noted that SEQ ID Numbers 2, 4, 6, 8, 10, 12, 14, 16, 23, 25 and 27 repeat the amino acid sequences listed with the nucleotide sequences of SEQ ID Numbers 1, 3, 5, 7, 9, 11, 13, 15, 22, 24 and 26, respectively.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BIOGEN, INC.
NEW ENGLAND MEDICAL CENTER HOSPITALS, INC.
BARSOUM, James G. (US only)
ANDROPHY, Elliot J. (US only)

(ii) TITLE OF INVENTION: PAPILLOMAVIRUS E2 TRANS-ACTIVATION
REPRESSORS

(iii) NUMBER OF SEQUENCES: 30

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: FISH & NEAVE
(B) STREET: 875 Third Avenue
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10022-6250

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Haley Jr., James F.
(B) REGISTRATION NUMBER: 27,794
(C) REFERENCE/DOCKET NUMBER: B156CIP

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212)715-0600
(B) TELEFAX: (212)715-0673
(C) TELEX: 14-8367

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 381 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
- (B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC	48
Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT	96
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA	144
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	
35 40 45	
GGA ACT GCT AAC CAG GTA AAG TGC TAT CGC TTT CGG GTG AAA AAG AAC	192
Gly Thr Ala Asn Gln Val Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn	
50 55 60	
CAT AGA CAT CGC TAC GAG AAC TGC ACC ACC ACC TGG TTC ACA GTT GCT	240
His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala	
65 70 75 80	
GAC AAC GGT GCT GAA AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT	288
Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe	
85 90 95	
GGA TCG CCA AGT CAA AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT	336
Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro	
100 105 110	
CCT GGA ATG AAC ATT TCC GGC TTT ACA GCC AGC TTG GAC TTC	378
Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe	
115 120 125	
TGA	381

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp
1 5 10 15

Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp
20 25 30

Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser
35 40 45

Gly Thr Ala Asn Gln Val Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn
50 55 60

His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala
65 70 75 80

Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe
85 90 95

Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro
100 105 110

Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe
115 120 125

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 381 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
- (B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC	48
Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT	96
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA	144
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	
35 40 45	
GGA ACT GCT AAC CTG GTA AAG TGC TAT CGC TTT CGG GTG AAA AAG AAC	192
Gly Thr Ala Asn Leu Val Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn	
50 55 60	
CAT AGA CAT CGC TAC GAG AAC TGC ACC ACC ACC TGG TTC ACA GTT GCT	240
His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala	
65 70 75 80	
GAC AAC GGT GCT GAA AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT	288
Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe	
85 90 95	
GGA TCG CCA AGT CAA AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT	336
Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro	
100 105 110	
CCT GGA ATG AAC ATT TCC GGC TTT ACA GCC AGC TTG GAC TTC	378
Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe	
115 120 125	
TGA	381

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	

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Gly Ph His Leu Leu Lys Aia Gly Gly Ser Cys Phe Ala Leu Ile Ser
 35 40 45

Gly Thr Ala Asn Leu Val Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn
 50 55 60

His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala
 65 70 75 80

Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe
 85 90 95

Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro
 100 105 110

Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe
 115 120 125

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 381 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC	48
Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT	96
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA	144
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	
35 40 45	
GGA ACT GCT AAC CAG GTA ATG TGC TAT CGC TTT CGG GTG AAA AAG AAC	192
Gly Thr Ala Asn Gln Val Met Cys Tyr Arg Phe Arg Val Lys Lys Asn	
50 55 60	

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Pro	Val	Asp	Leu	Ala	Ser	Arg	Gln	Glu	Glu	Glu	Gln	Ser	Pro	Asp	
1														15	
Ser	Thr	Glu	Glu	Glu	Pro	Val	Thr	Leu	Pro	Arg	Arg	Thr	Thr	Asn	Asp
					20				25					30	
Gly	Phe	His	Leu	Leu	Lys	Ala	Gly	Gly	Ser	Cys	Phe	Ala	Leu	Ile	Ser
						35			40					45	
Gly	Thr	Ala	Asn	Gln	Val	Met	Cys	Tyr	Arg	Phe	Arg	Val	Lys	Lys	Asn
						50			55					60	
His	Arg	His	Arg	Tyr	Glu	Asn	Cys	Thr	Thr	Thr	Trp	Phe	Thr	Val	Ala
								70			75				80
Asp	Asn	Gly	Ala	Glu	Arg	Gln	Gly	Gln	Ala	Gln	Ile	Leu	Ile	Thr	Phe
									85		90			95	
Gly	Ser	Pro	Ser	Gln	Arg	Gln	Asp	Phe	Leu	Lys	His	Val	Pro	Leu	Pro
									100		105			110	
Pro	Gly	Met	Asn	Ile	Ser	Gly	Phe	Thr	Ala	Ser	Leu	Asp	Phe		
									115		120			125	

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 381 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
- (B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC	48
Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT	96
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA	144
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	
35 40 45	
GGA ACT GCT AAC CAG GTA AAG TTC TAT CGC TTT CGG GTG AAA AAG AAC	192
Gly Thr Ala Asn Gln Val Lys Phe Tyr Arg Phe Arg Val Lys Lys Asn	
50 55 60	
CAT AGA CAT CGC TAC GAG AAC TGC ACC ACC ACC TGG TTC ACA GTT GCT	240
His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala	
65 70 75 80	
GAC AAC GGT GCT GAA AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT	288
Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe	
85 90 95	
GGA TCG CCA AGT CAA AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT	336
Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro	
100 105 110	

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CCT GGA ATG AAC ATT TCC GGC TTT ACA GCC AGC TTG GAC TTC
 Pro Gly Met Asn Ile S r Gly Phe Thr Ala Ser Leu Asp Phe
 115 120 125

378

TGA

381

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp
 1 5 10 15

Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp
 20 25 30

Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser
 35 40 45

Gly Thr Ala Asn Gln Val Lys Phe Tyr Arg Phe Arg Val Lys Lys Asn
 50 55 60

His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala
 65 70 75 80

Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe
 85 90 95

Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro
 100 105 110

Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe
 115 120 125

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 381 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
- (B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC	48
Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT	96
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA	144
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	
35 40 45	
GGA ACT GCT AAC CAG GTA AAG CGC TAT CGC TTT CGG GTG AAA AAG AAC	192
Gly Thr Ala Asn Gln Val Lys Arg Tyr Arg Phe Arg Val Lys Lys Asn	
50 55 60	
CAT AGA CAT CGC TAC GAG AAC TGC ACC ACC ACC TGG TTC ACA GTT GCT	240
His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala	
65 70 75 80	
GAC AAC GGT GCT GAA AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT	288
Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe	
85 90 95	
GGA TCG CCA AGT CAA AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT	336
Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro	
100 105 110	
CCT GGA ATG AAC ATT TCC GGC TTT ACA GCC AGC TTG GAC TTC	378
Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe	
115 120 125	
TGA	381

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln Ser Pro Asp
1 5 10 15

Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp
20 25 30

Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser
35 40 45

Gly Thr Ala Asn Gln Val Lys Arg Tyr Arg Phe Arg Val Lys Lys Asn
50 55 60

His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala
65 70 75 80

Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe
85 90 95

Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro
100 105 110

Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe
115 120 125

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 381 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
- (B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Gln Ser Pro Asp	48
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	96
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	144
35 40 45	
GGA ACT GCT AAC CAG GTA AAG TAC TAT CGC TTT CGG GTG AAA AAG AAC Gly Thr Ala Asn Gln Val Lys Tyr Tyr Arg Phe Arg Val Lys Lys Asn	192
50 55 60	
CAT AGA CAT CGC TAC GAG AAC TGC ACC ACC TGG TTC ACA GTT GCT His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala	240
65 70 75 80	
GAC AAC GGT GCT GAA AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe	288
85 90 95	
GGA TCG CCA AGT CAA AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro	336
100 105 110	
CCT GGA ATG AAC ATT TCC GGC TTT ACA GCC AGC TTG GAC TTC Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe	378
115 120 125	
TGA	381

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	

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Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser
 35 40 45

Gly Thr Ala Asn Gln Val Lys Tyr Tyr Arg Phe Arg Val Lys Lys Asn
 50 55 60

His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala
 65 70 75 80

Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe
 85 90 95

Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro
 100 105 110

Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe
 115 120 125

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 381 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
- (B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..378

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC	48
Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Gln Ser Pro Asp	
1 5 10 15	
TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT	96
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp	
20 25 30	
GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA	144
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser	
35 40 45	

GGA ACT GCT AAC CAG GTA AAG TGC TAT CGC TTT CTG GTG AAA AAG AAC Gly Thr Ala Asn Gln Val Lys Cys Tyr Arg Phe Leu Val Lys Lys Asn 50 55 60	192
CAT AGA CAT CGC TAC GAG AAC TGC ACC ACC ACC TGG TTC ACA GTT GCT His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala 65 70 75 80	240
GAC AAC GGT GCT GAA AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe 85 90 95	288
GGA TCG CCA AGT CAA AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro 100 105 110	336
CCT GGA ATG AAC ATT TCC GGC TTT ACA GCC AGC TTG GAC TTC Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe 115 120 125	378
TGA	381

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Gln Ser Pro Asp 1 5 10 15
Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg Thr Thr Asn Asp 20 25 30
Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser 35 40 45
Gly Thr Ala Asn Gln Val Lys Cys Tyr Arg Phe Leu Val Lys Lys Asn 50 55 60
His Arg His Arg Tyr Glu Asn Cys Thr Thr Trp Phe Thr Val Ala 65 70 75 80
Asp Asn Gly Ala Glu Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe 85 90 95
Gly Ser Pro Ser Gln Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro 100 105 110

Pro Gly Met Asn Ile Ser Gly Phe Thr Ala Ser Leu Asp Phe
115 120 125

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 222 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bovine papillomavirus
(B) STRAIN: Type 1

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 1..219

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTA AAG TGC TAT CGC TTT CCG GTG AAA AAG AAC CAT AGA CAT CGC TAC
 Val Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn His Arg His Arg Tyr
 1 5 10 15

48

GAG AAC TGC ACC ACC ACC TGG TTC ACA GTT GCT GAC AAC GGT GCT GAA
 Glu Asn Cys Thr Thr Thr Trp Phe Thr Val Ala Asp Asn Gly Ala Glu
 20 25 30

96

AGA CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT GGA TCG CCA AGT CAA
 Arg Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe Gly Ser Pro Ser Gln
 35 40 45

144

AGG CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT CCT GGA ATG AAC ATT
 Arg Gln Asp Phe Leu Lys His Val Pro Leu Pro Pro Gly Met Asn Ile
 50 55 60

192

TCC GGC TTT ACA GCC AGC TTG GAC TTC TGA
Ser Gly Phe Thr Ala Ser Leu Asp Phe
65 70

222

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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Val	Lys	Cys	Tyr	Arg	Phe	Arg	Val	Lys	Lys	Asn	His	Arg	His	Arg	Tyr
1				5				10						15	
Glu	Asn	Cys	Thr	Thr	Trp	Phe	Thr	Val	Ala	Asp	Asn	Gly	Ala	Glu	
	20						25						30		
Arg	Gln	Gly	Gln	Ala	Gln	Ile	Leu	Ile	Thr	Phe	Gly	Ser	Pro	Ser	Gln
	35						40						45		
Arg	Gln	Asp	Phe	Leu	Lys	His	Val	Pro	Leu	Pro	Pro	Gly	Met	Asn	Ile
	50					55							60		
Ser	Gly	Phe	Thr	Ala	Ser	Leu	Asp	Phe							
	65					70									

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCAACTAGT CCCAAG

16

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTCGAGAAGC TTGACGGATC CG

22

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGCAGAGCTC TTCTGAAC TGC CTAGGCTTAA

30

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GATCCTTTGC CGCCAC

16

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAAACGGCGG TGGTAC

16

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 258 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..255

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG GCT AGC AAC ACT ACA CCC ATA GTA CAT TTA AAA GGT GAT GCT AAT Met Ala Ser Asn Thr Thr Pro Ile Val His Leu Lys Gly Asp Ala Asn	48
1 5 10 15	
ACT TTA AAA TGT TTA AGA TAT AGA TTT AAA AAG CAT TGT ACA TTG TAT Thr Leu Lys Cys Leu Arg Tyr Arg Phe Lys Lys His Cys Thr Leu Tyr	96
20 25 30	
ACT GCA GTG TCG TCT ACA TGG CAT TGG ACA GGA CAT AAT GTA AAA CAT Thr Ala Val Ser Ser Thr Trp His Trp Thr Gly His Asn Val Lys His	144
35 40 45	
AAA AGT GCA ATT GTT ACA CTT ACA TAT GAT AGT GAA TGG CAA CGT GAC Lys Ser Ala Ile Val Thr Leu Thr Tyr Asp Ser Glu Trp Gln Arg Asp	192
50 55 60	
CAA TTT TTG TCT CAA GTT AAA ATA CCA AAA ACT ATT ACA GTG TCT ACT Gln Phe Leu Ser Gln Val Lys Ile Pro Lys Thr Ile Thr Val Ser Thr	240
65 70 75 80	
GGA TTT ATG TCT ATA TGA Gly Phe Met Ser Ile	258
85	

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 85 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Ala Ser Asn Thr Thr Pro Ile Val His Leu Lys Gly Asp Ala Asn	
1 5 10 15	
Thr Leu Lys Cys Leu Arg Tyr Arg Phe Lys Lys His Cys Thr Leu Tyr	
20 25 30	
Thr Ala Val Ser Ser Thr Trp His Trp Thr Gly His Asn Val Lys His	
35 40 45	
Lys Ser Ala Ile Val Thr Leu Thr Tyr Asp Ser Glu Trp Gln Arg Asp	
50 55 60	
Gln Phe Leu Ser Gln Val Lys Ile Pro Lys Thr Ile Thr Val Ser Thr	
65 70 75 80	
Gly Phe Met Ser Ile	
85	

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 372 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..369

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATG GTA CCA GAC ACC GGA AAC CCC TGC CAC ACC ACT AAG TTG TTG CAC	48
Met Val Pro Asp Thr Gly Asn Pro Cys His Thr Thr Lys Leu Leu His	
1 5 10 15	
AGA GAC TCA GTG GAC AGT GCT CCA ATC CTC ACT GCA TTT AAC AGC TCA	96
Arg Asp Ser Val Asp Ser Ala Pro Ile Leu Thr Ala Phe Asn Ser Ser	
20 25 30	
CAC AAA GGA CGG ATT AAC TGT AAT AGT AAC ACT ACA CCC ATA GTA CAT	144
His Lys Gly Arg Ile Asn Cys Asn Ser Asn Thr Thr Pro Ile Val His	
35 40 45	
TTA AAA GGT GAT GCT AAT ACT TTA AAA TGT TTA AGA TAT AGA TTT AAA	192
Leu Lys Gly Asp Ala Asn Thr Leu Lys Cys Leu Arg Tyr Arg Phe Lys	
50 55 60	
AAG CAT TGT ACA TTG TAT ACT GCA GTG TCG TCT ACA TGG CAT TGG ACA	240
Lys His Cys Thr Leu Tyr Thr Ala Val Ser Ser Thr Trp His Trp Thr	
65 70 75 80	
GGA CAT AAT GTA AAA CAT AAA AGT GCA ATT GTT ACA CTT ACA TAT GAT	288
Gly His Asn Val Lys His Lys Ser Ala Ile Val Thr Leu Thr Tyr Asp	
85 90 95	
AGT GAA TGG CAA CGT GAC CAA TTT TTG TCT CAA GTT AAA ATA CCA AAA	336
Ser Glu Trp Gln Arg Asp Gln Phe Leu Ser Gln Val Lys Ile Pro Lys	
100 105 110	
ACT ATT ACA GTG TCT ACT GGA TTT ATG TCT ATA TGA	372
Thr Ile Thr Val Ser Thr Gly Phe Met Ser Ile	
115 120	

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 123 amino acids
- (B) TYPE: amino acid

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met	Val	Pro	Asp	Thr	Gly	Asn	Pro	Cys	His	Thr	Thr	Lys	Leu	Leu	His
1					5				10				15		
Arg	Asp	Ser	Val	Asp	Ser	Ala	Pro	Ile	Leu	Thr	Ala	Phe	Asn	Ser	Ser
			20						25				30		
His	Lys	Gly	Arg	Ile	Asn	Cys	Asn	Ser	Asn	Thr	Thr	Pro	Ile	Val	His
				35				40				45			
Leu	Lys	Gly	Asp	Ala	Asn	Thr	Leu	Lys	Cys	Leu	Arg	Tyr	Arg	Phe	Lys
			50				55				60				
Lys	His	Cys	Thr	Leu	Tyr	Thr	Ala	Val	Ser	Ser	Thr	Trp	His	Trp	Thr
			65			70			75			80			
Gly	His	Asn	Val	Lys	His	Lys	Ser	Ala	Ile	Val	Thr	Leu	Thr	Tyr	Asp
				85				90			95				
Ser	Glu	Trp	Gln	Arg	Asp	Gln	Phe	Leu	Ser	Gln	Val	Lys	Ile	Pro	Lys
			100				105				110				
Thr	Ile	Thr	Val	Ser	Thr	Gly	Phe	Met	Ser	Ile					
				115				120							

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 939 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..936

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATG	GAA	CCG	GTC	GAC	CCG	CGT	CTG	GAA	CCA	TGG	AAA	CAC	CCC	GGG	TCC
Met															
1					5				10				15		

48

CAG	CCG	AAA	ACC	GCG	TGC	ACC	AAC	TGC	TAC	TGC	AAA	AAA	TGC	TGC	TTC
Gln															
20									25				30		

96

CAC TGC CAG GTT TGC TTC ATC ACC AAA GCC CTA GGT ATC TCT TAC GGC His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly 35 40 45	144
CGT AAA AAA CGT CGT CAG CGA CGT CGT CCG CCG CAG GGA TCT TCC ATG Arg Lys Lys Arg Arg Gln Arg Arg Pro Pro Gln Gly Ser Ser Met 50 55 60	192
GCC GGT GCT GGA CGC ATT TAC TAT TCT CGC TTT GGT GAC GAG GCA GCC Ala Gly Ala Gly Arg Ile Tyr Tyr Ser Arg Phe Gly Asp Glu Ala Ala 65 70 75 80	240
AGA TTT AGT ACA ACA GGG CAT TAC TCT GTA AGA GAT CAG GAC AGA GTG Arg Phe Ser Thr Thr Gly His Tyr Ser Val Arg Asp Gln Asp Arg Val 85 90 95	288
TAT GCT GGT GTC TCA TCC ACC TCT TCT GAT TTT AGA GAT CGC CCA GAC Tyr Ala Gly Val Ser Ser Thr Ser Asp Phe Arg Asp Arg Pro Asp 100 105 110	336
GGA GTC TGG GTC GCA TCC GAA GGA CCT GAA GGA GAC CCT GCA GGA AAA Gly Val Trp Val Ala Ser Glu Gly Pro Glu Gly Asp Pro Ala Gly Lys 115 120 125	384
GAA GCC GAG CCA GCC CAG CCT GTC TCT TCT TTG CTC GGC TCC CCC GCC Glu Ala Glu Pro Ala Gln Pro Val Ser Ser Leu Leu Gly Ser Pro Ala 130 135 140	432
TGC GGT CCC ATC AGA GCA GGC CTC GGT TGG GTA CGG GAC GGT CCT CGC Cys Gly Pro Ile Arg Ala Gly Leu Gly Trp Val Arg Asp Gly Pro Arg 145 150 155 160	480
TCG CAC CCC TAC AAT TTT CCT GCA GGC TCG GGG GGC TCT ATT CTC CGC Ser His Pro Tyr Asn Phe Pro Ala Gly Ser Gly Gly Ser Ile Leu Arg 165 170 175	528
TCT TCC TCC ACC CCG GTG CAG GGC ACG GTA CCG GTG GAC TTG GCA TCA Ser Ser Ser Thr Pro Val Gln Gly Thr Val Pro Val Asp Leu Ala Ser 180 185 190	576
AGG CAG GAA GAA GAG GAG CAG TCG CCC GAC TCC ACA GAG GAA GAA CCA Arg Gln Glu Glu Glu Gln Ser Pro Asp Ser Thr Glu Glu Glu Pro 195 200 205	624
GTG ACT CTC CCA AGG CGC ACC ACC AAT GAT GGA TTC CAC CTG TTA AAG Val Thr Leu Pro Arg Arg Thr Thr Asn Asp Gly Phe His Leu Leu Lys 210 215 220	672
GCA GGA GGG TCA TGC TTT GCT CTA ATT TCA GGA ACT GCT AAC CAG GTA Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser Gly Thr Ala Asn Gln Val 225 230 235 240	720
AAG TGC TAT CGC TTT CGG GTG AAA AAG AAC CAT AGA CAT CGC TAC GAG Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn His Arg His Arg Tyr Glu 245 250 255	768

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AAC TGC ACC ACC TGG TTC ACA GTT GCT GAC AAC GGT GCT GAA AGA	816
Asn Cys Thr Thr Trp Phe Thr Val Ala Asp Asn Gly Ala Glu Arg	
260 265 270	
CAA GGA CAA GCA CAA ATA CTG ATC ACC TTT GGA TCG CCA AGT CAA AGG	864
Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe Gly Ser Pro Ser Gln Arg	
275 280 285	
CAA GAC TTT CTG AAA CAT GTA CCA CTA CCT CCT GGA ATG AAC ATT TCC	912
Gln Asp Phe Leu Lys His Val Pro Leu Pro Pro Gly Met Asn Ile Ser	
290 295 300	
GGC TTT ACA GCC AGC TTG GAC TTC TGA	939
Gly Phe Thr Ala Ser Leu Asp Phe	
305 310	

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 312 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser	
1 5 10 15	
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe	
20 25 30	
His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly	
35 40 45	
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Ser Met	
50 55 60	
Ala Gly Ala Gly Arg Ile Tyr Tyr Ser Arg Phe Gly Asp Glu Ala Ala	
65 70 75 80	
Arg Phe Ser Thr Thr Gly His Tyr Ser Val Arg Asp Gln Asp Arg Val	
85 90 95	
Tyr Ala Gly Val Ser Ser Thr Ser Ser Asp Phe Arg Asp Arg Pro Asp	
100 105 110	
Gly Val Trp Val Ala Ser Glu Gly Pro Glu Gly Asp Pro Ala Gly Lys	
115 120 125	
Glu Ala Glu Pro Ala Gln Pro Val Ser Ser Leu Leu Gly Ser Pro Ala	
130 135 140	

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Cys Gly Pro Ile Arg Ala Gly Leu Gly Trp Val Arg Asp Gly Pro Arg
 145 150 155 160

Ser His Pro Tyr Asn Ph Pro Ala Gly Ser Gly Gly Ser Ile Leu Arg
 165 170 175

Ser Ser Ser Thr Pro Val Gln Gly Thr Val Pro Val Asp Leu Ala Ser
 180 185 190

Arg Gln Glu Glu Glu Gln Ser Pro Asp Ser Thr Glu Glu Glu Pro
 195 200 205

Val Thr Leu Pro Arg Arg Thr Thr Asn Asp Gly Phe His Leu Leu Lys
 210 215 220

Ala Gly Gly Ser Cys Phe Ala Leu Ile Ser Gly Thr Ala Asn Gln Val
 225 230 235 240

Lys Cys Tyr Arg Phe Arg Val Lys Lys Asn His Arg His Arg Tyr Glu
 245 250 255

Asn Cys Thr Thr Trp Phe Thr Val Ala Asp Asn Gly Ala Glu Arg
 260 265 270

Gln Gly Gln Ala Gln Ile Leu Ile Thr Phe Gly Ser Pro Ser Gln Arg
 275 280 285

Gln Asp Phe Leu Lys His Val Pro Leu Pro Pro Gly Met Asn Ile Ser
 290 295 300

Gly Phe Thr Ala Ser Leu Asp Phe
 305 310

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTCCCATGGC TAGCAACACT ACACCC

26

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTCCCCATGGT ACCAGACACC GGAAACC

27

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGGGGATCCT CATATAGACA TAAATCC

27

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CLAIMS

1. An E2 trans-activation repressor comprising a polypeptide having an amino acid sequence homologous to the native E2 DNA binding domain (SEQ ID NO:1), said polypeptide being capable of forming inactive heterodimers with native E2 protein and said inactive heterodimers being incapable of binding to E2 DNA binding sites.

2. An E2 trans-activation repressor comprising a polypeptide fragment of the native E2 DNA binding domain, said fragment being capable of forming inactive heterodimers with native E2 protein and said inactive heterodimers being incapable of binding to E2 DNA binding sites.

3. The E2 trans-activation repressor according to claim 1, said repressor consisting essentially of a polypeptide having an amino acid sequence homologous to the native E2 DNA binding domain (SEQ ID NO:1), said polypeptide being capable of forming inactive heterodimers with native E2 protein and said inactive heterodimers being incapable of binding to E2 DNA binding sites.

4. The E2 trans-activation repressor according to claim 2, said repressor consisting essentially of a polypeptide fragment of the native E2 DNA binding domain, said fragment being capable of forming inactive heterodimers with native E2 protein and said inactive heterodimers being incapable of binding to E2 DNA binding sites.

5. The E2 trans-activation repressor according to claim 1, wherein said amino acid sequence

is selected from the group consisting of the amino acid sequence defined by SEQ ID NO:3, the amino acid sequence defined by SEQ ID NO:5, the amino acid sequence defined by SEQ ID NO:7, the amino acid sequence defined by SEQ ID NO:9, the amino acid sequence defined by SEQ ID NO:11, the amino acid sequence defined by SEQ ID NO:13, the amino acid sequence defined by SEQ ID NO:23 and the amino acid sequence defined by SEQ ID NO:25.

6. The E2 trans-activation repressor according to claim 2, wherein said repressor consists essentially of a polypeptide of the formula selected from the group consisting of:

- a) AA₃₃₈ to AA₄₁₀ of the native BPV1 E2 protein (SEQ ID NO:15); and
- b) AA₂₈₃ to AA₃₆₅ of the native HPV16 E2 protein (SEQ ID NO:23).

7. An E2 trans-activation repressor comprising a non-peptide moiety, said moiety being capable of specifically forming inactive complexes with full-length native E2 polypeptides.

8. A DNA sequence encoding an E2 trans-activation repressor having an amino acid sequence homologous to the native E2 DNA binding domain (SEQ ID NO:1), said repressor being capable of forming inactive heterodimers with native E2 protein and said inactive heterodimers being incapable of binding to E2 DNA binding sites, wherein said DNA sequence comprises a DNA sequence selected from the group consisting of:

- a) SEQ ID NO:3;
- b) SEQ ID NO:5;
- c) SEQ ID NO:7;

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d) SEQ ID NO:9;
e) SEQ ID NO:11;
f) SEQ ID NO:13;
g) SEQ ID NO:22;
h) SEQ ID NO:24; and
i) DNA sequences which encode the amino acid sequence encoded by any one of the foregoing DNA sequences.

9. A DNA sequence encoding an E2 trans-activation repressor comprising a polypeptide fragment of the native E2 DNA binding domain, said fragment being capable of forming inactive heterodimers with native E2 protein and said inactive heterodimers being incapable of binding to E2 DNA binding sites, wherein said DNA sequence comprises a DNA sequence selected from the group consisting of:

a) DNA sequences which are a fragment of the native E2 DNA binding domain and
b) DNA sequences which encode the amino acid sequence encoded by any one of the foregoing DNA sequences.

10. The DNA sequence according to claim 8, selected from the group consisting of the DNA inserts of: pEC337L, pEC339M, pEC340F, pEC340R, pEC340Y, pEC344L, pHE2-85 and pHE2-123.

11. A recombinant DNA molecule comprising a DNA sequence according to claim 8 or 9.

12. The recombinant DNA molecule according to claim 11, wherein said DNA sequence is operatively linked to an expression control sequence.

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13. The recombinant DNA mol cul according to claim 12, wherein the expr ssi n control sequence is selected from the group consisting of the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, the promoters of th yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and combinations thereof.

14. The recombinant DNA molecule according to claim 13, said molecule being selected from the group consisting of pEC337L, pEC339M, pEC340F, pEC340R, pEC340Y, pEC344L, pHE2-85 and pHE2-123.

15. A unicellular host transformed with a recombinant DNA molecule according to any one of claims 11 to 14.

16. The transformed host according to claim 15, wherein said unicellular host is selected from the group consisting of E.coli, Pseudomonas, Bacillus, Streptomyces, Saccharomyces and other fungi, plant cells in culture, insect cells in culture, animal cells in culture and human cells in culture.

17. The transformed host according to claim 16, wherein said host is selected from the group consisting of E.coli DH5/pEC337L, E.coli DH5/pEC339M,

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E.coli DH5/pEC340F, E.coli DH5/pEC340R, E.coli DH5/pEC340Y, E.c li DH5/pEC344L, E.coli pHE2-85, E.coli pHE2-123 and E.coli pFTE501.

18. A process for producing an E2 trans-activation repressor, said process comprising the st ps of:

- a) culturing a transformed host according to claim 15; and
- b) recovering the E2 trans-activation repressor from said culture.

19. The E2 trans-activation repressor according to claim 1 or 2, said repressor further comprising a lipophilic moiety.

20. The E2 trans-activation repressor according to claim 19, wherein said lipophilic moiety is a fatty acid molecule.

21. The E2 trans-activation repressor according to claim 1 or 2, said repressor further comprising a basic polymer.

22. The E2 trans-activation repressor according to claim 21, wherein said polymer is selected from the group consisting of polyarginine and polylysine.

23. An E2 trans-activation repressor fusion protein comprising an E2 trans-activation repressor according to any one of claims 1 to 3 and a transport moiety.

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24. The E2 trans-activation repressor fusion protein according to claim 23, wherein said transport moiety is selected from the group consisting of bacterial hemolysins, cell entry components of bacterial toxins, viral receptors, cell receptors, cell ligands, bacterial immunogens, parasitic immunogens, viral immunogens, immunoglobulins or fragments thereof that bind to target molecules, cytokines, growth factors, colony stimulating factors, tat protein and hormones.

25. The E2 trans-activation repressor fusion protein according to claim 23 or 24, wherein the amino terminus of the E2 repressor is fused to the carboxy terminus of the transport moiety.

26. A pharmaceutically acceptable composition for treating papillomavirus infection comprising a pharmaceutically effective amount of an E2 trans-activation repressor according to any one of claims 1 to 3 and a pharmaceutically acceptable carrier.

27. A pharmaceutically acceptable composition for treating papillomavirus infection comprising an E2 trans-activation repressor fusion protein according to claim 23 or 24 and a pharmaceutically acceptable carrier.

28. A pharmaceutically acceptable composition for treating papillomavirus infection comprising an E2 trans-activation repressor according to claim 7 and a pharmaceutically acceptable carrier.

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29. A method for treating papillomavirus infection in a patient comprising the step of administering to the patient a pharmaceutically effective amount of a pharmaceutically acceptable composition according to any one of claims 26 to 28.

30. The method according to claim 29, wherein said pharmaceutically effective amount is between about 1 and 1000 mg/kg body weight per day when said composition is administered parenterally.

31. The method according to claim 29, wherein said pharmaceutically effective amount is between about 1 and 1000 µg/ml when said composition is administered topically.

32. A method for treating papillomavirus-infected cells comprising the step of introducing into said cells an E2 trans-activation repressor according to any one of claims 1, 2, 3 or 7.

33. A method for treating papillomavirus-infected cells comprising the step of introducing into said cells a DNA sequence according to any one of claims 8 to 10.

34. The method according to claim 33, wherein said DNA sequence is introduced into said cells by treating said cells to allow uptake of said DNA.

35. The method according to claim 33, wherein said DNA sequence is introduced into said cells by electroporation.

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36. The method according to claim 33, wherein said DNA sequence is introduced into said cells by direct injection.

37. The method according to claim 33, wherein said DNA sequence is introduced into said cells by means of a defective recombinant virus.

38. The method according to claim 37, said method comprising the steps of:

- a) transcribing said DNA sequence into an RNA sequence; and
- b) incorporating said RNA sequence into a defective recombinant retrovirus.

39. A method for isolating mutations yielding E2 homologous polypeptides that repress trans-activation by native E2 proteins comprising the steps of:

- a) producing a population of mutant DNA fragments by mutagenizing a DNA fragment encoding the amino acids of the native minimal DNA binding domain of a papillomavirus E2 protein;
- b) operatively linking said population of DNA fragments to expression control sequences;
- c) inserting said population of DNA fragments operatively linked to expression control sequences into a vector;
- d) transforming a unicellular host with said vector, said host comprising a full-length native E2 gene operatively linked to expression control sequences and a reporter gene whose transcription is activated by dimers comprising full-length native E2 proteins;

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-) screening for clones of transformed host cells that do not display E2 trans-activation;
- f) testing E2 homologous polypeptides from said clones for their ability to dimerize with themselves;
- g) testing said E2 homologous polypeptides for their ability to bind to E2 DNA binding sites;
- h) testing said E2 homologous polypeptides for their ability to repress transcriptional activation by full-length native E2 protein in a eukaryotic cell.

40. The use of a polypeptide comprising the E2 protein dimerization region to design E2 trans-activation repressors.

41. The use of a polypeptide consisting essentially of the E2 protein dimerization region to design E2 trans-activation repressors.

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PRO	VAL	ASP	LEU	ALA	SER	ARG	GLN	GLU	GLU	GLU	GLU	GLN
285												
SER	PRO	ASP	SER	<u>ser</u> THR	GLU	GLU	PRO	VAL	THR	LEU	PRO	
												310
ARG	ARG	THR	THR	ASN	<u>lys</u> ASP	GLY	PHE	HIS	LEU	LEU	LYS	ALA
GLY	GLY	SER	CYS	PHE	ALA	LEU	I ^{leu} E	SER	<u>val</u> GLY	THR	ALA	ASN
<u>leu</u> GLN	VAL	<u>met</u> LYS	<u>S,R,C,F,Y</u> CYS	TYR	ARG	PHE	<u>leu</u> ARC	VAL	LYS	<u>lys</u> ASN	<u>leu</u> HIS	
ARG	HIS	ARG	TYR	GLU	ASN	CYS	THR	THR	THR	<u>ser</u> TRP	PHE	THR
350												
VAL	ALA	ASP	<u>lys</u> ASN	GLY	ALA	GLU	<u>ile</u> ARC	GLN	GLY	<u>leu</u> GLN	<u>ser</u> ALA	
PHE	LEU	<u>ile</u> LYS	HIS	VAL	PRO	LEU	PRO	PRO	<u>val</u> GLY	<u>mp</u> ARG	GLN	ASP
390												
SER	GLY	PHE	THR	ALA	SER	<u>leu</u> ASP	PHE	<u>ile</u> MET	ASN	ILE		
												400

11 new a.a.

leu - 22 a.a.

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FIG. 1

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<u>Mutant</u>	<u>Alternate Designation</u>	<u>Amino Acid</u>	<u>Mutation</u>	<u>DNA Binding</u>	<u>Dimerization</u>	<u>Repression</u>
316Y	393	316	D→Y	+	-	-
317STOP	352	317	G→stop	-	-	-
333V	44	333	G→V	-	-	-
337L	294	337	Q→L	-	-	-
339M	388	339	K→M	-	-	-
340S	EA28	340	C→S	+	-	-
340R		340	C→R	-	-	-
340F		340	C→F	-	-	-
340G		340	C→G	+	-	-
340Y		340	C→Y	-	-	-
344L	38	344	R→L	-	-	-
360S	213W	360	W→S	super-shift		

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FIG. 2A

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<u>Mutant</u>	<u>Alternate Designation</u>	<u>Amino Acid</u>	<u>Mutation</u>	<u>DNA Binding</u>	<u>Dimerization</u>	<u>Repression</u>
366X/376L	354		366/376 N→Y, I→L	+	+	-
370I	10		370 R→I	+	+	(±)
386W	9		386 R→W	+	+	
399I	111		399 M→I	+	+	
402*	3812SS		402 4 aa insert	-	super-shift	
408*	36		402 Δ ; + 11aa	+	+	(±)
411	211		411 STOP→L	+	+	
374S/375L	3SLI		374, 375, 391 A→S, Q→L, K→I	super-shift	(±)	
391I						

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FIG. 2B

FIG. 3A

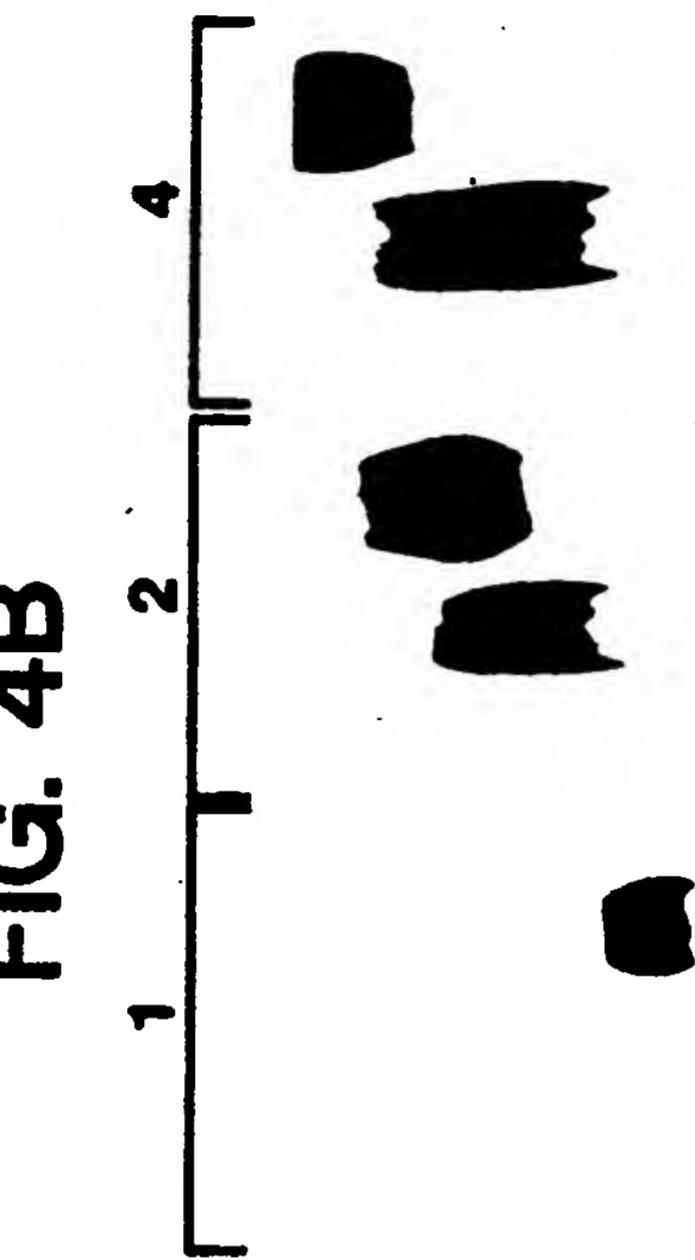
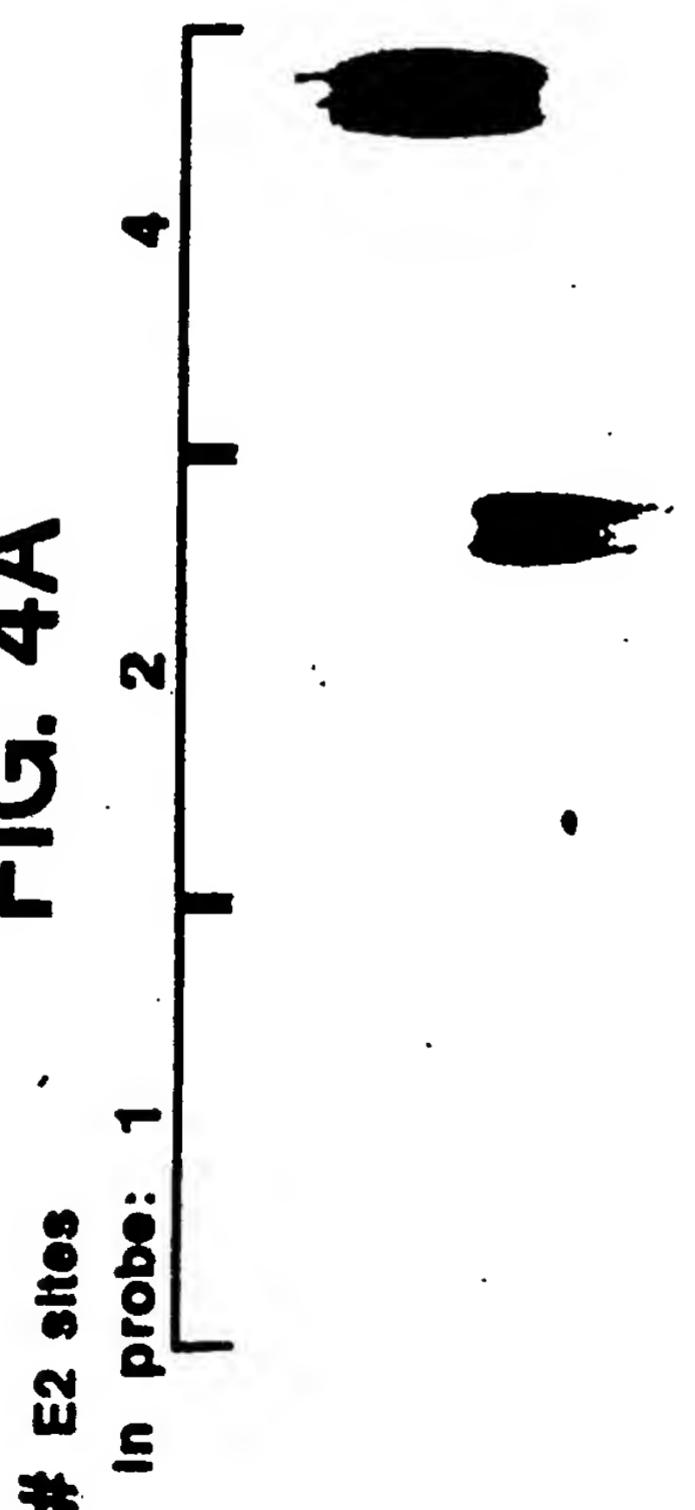
3B
FIG.

30
FIG.

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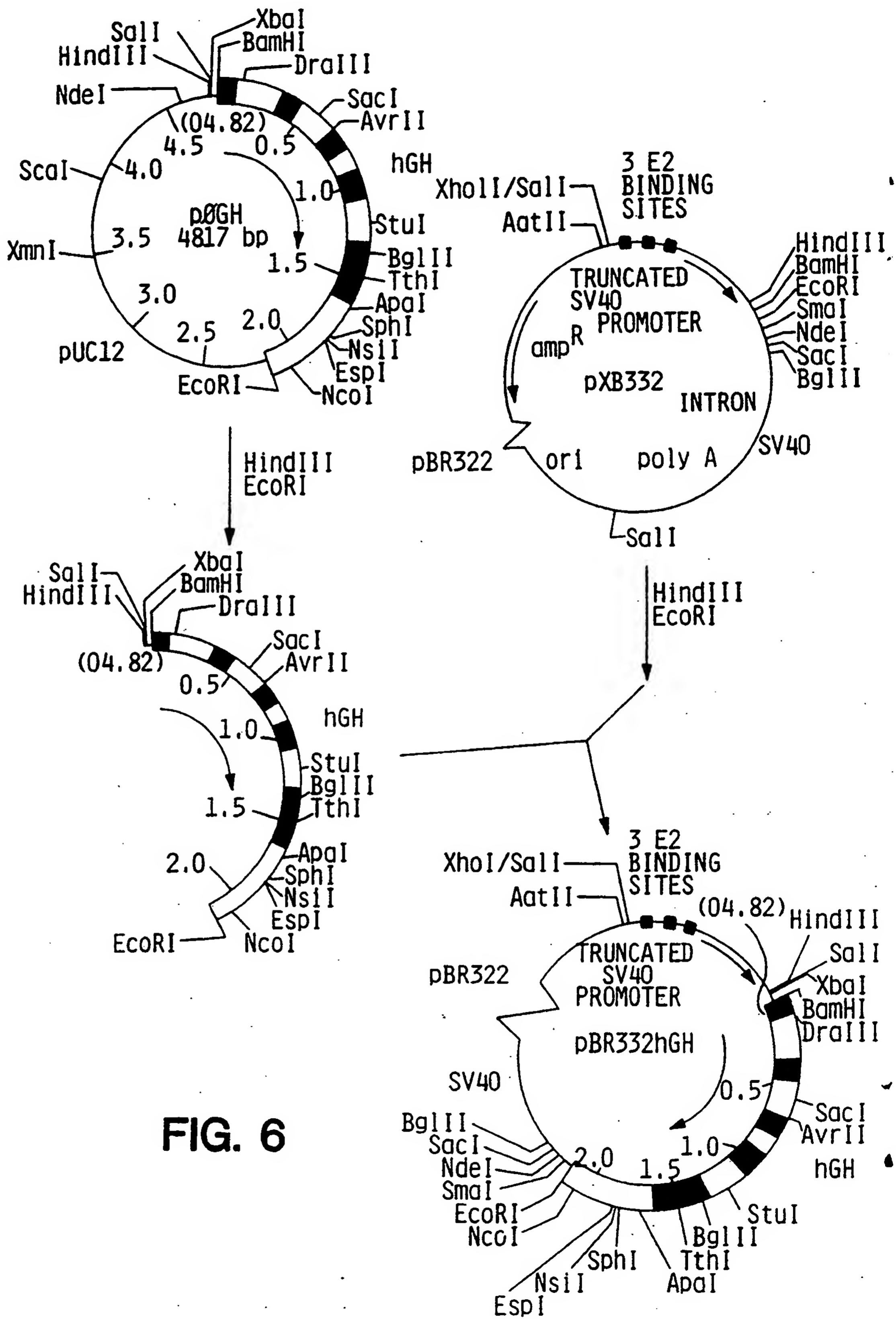
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FIG. 4B**FIG. 4A**# E2 sites
In probe: 1

P 1A 1B P 2A 2B P 4A 4B

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**FIG. 6**

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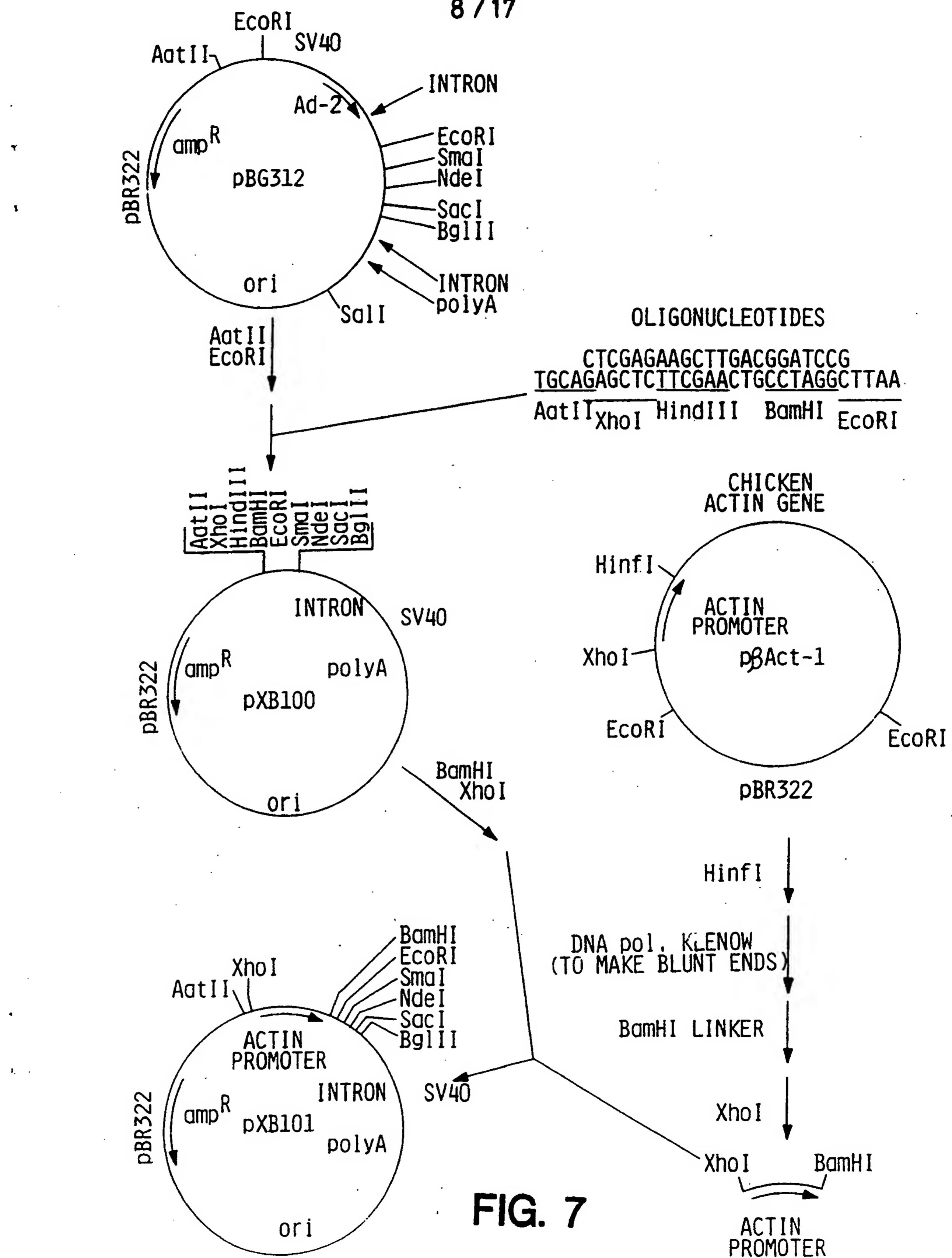


FIG. 7

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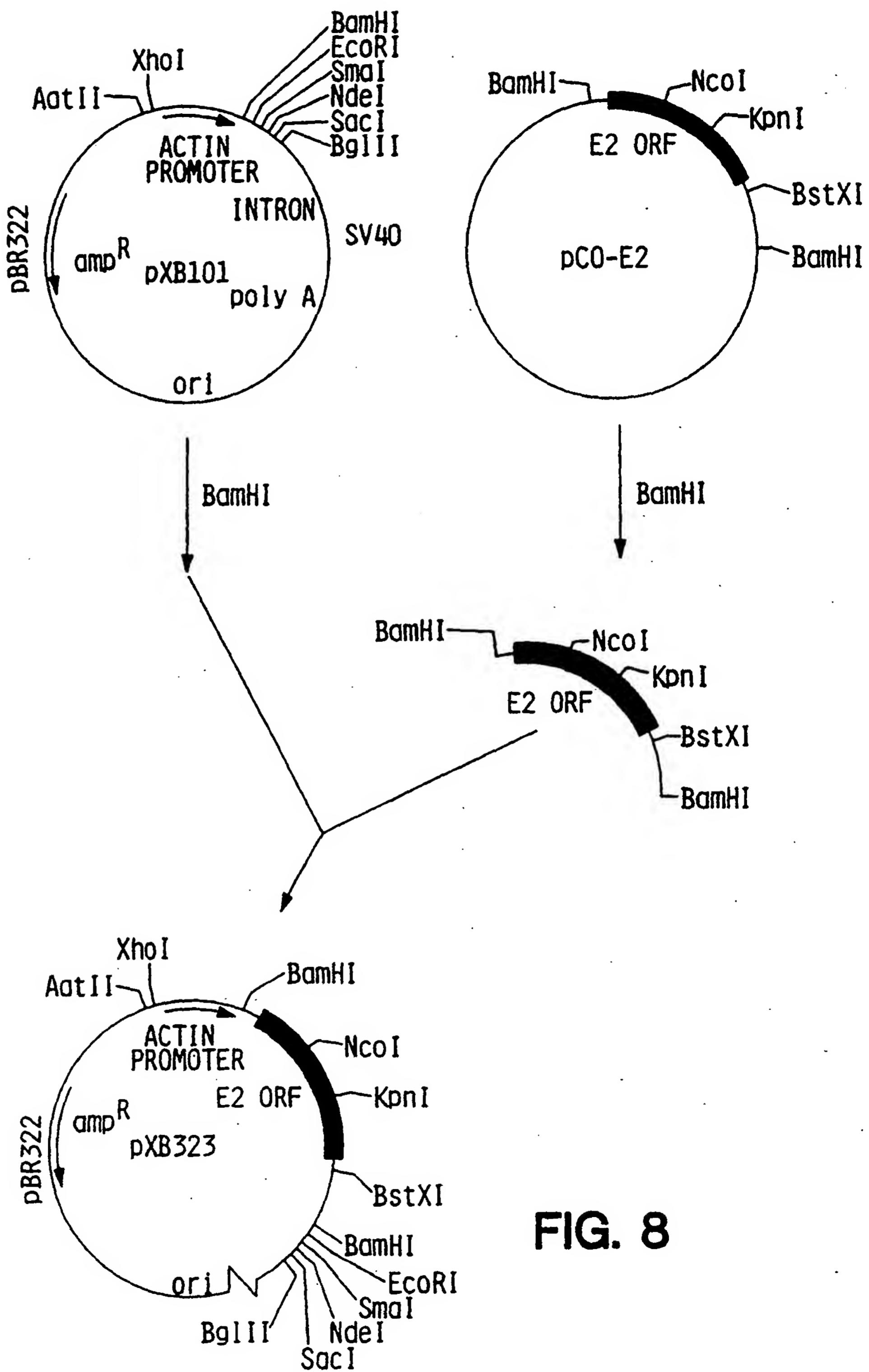


FIG. 8

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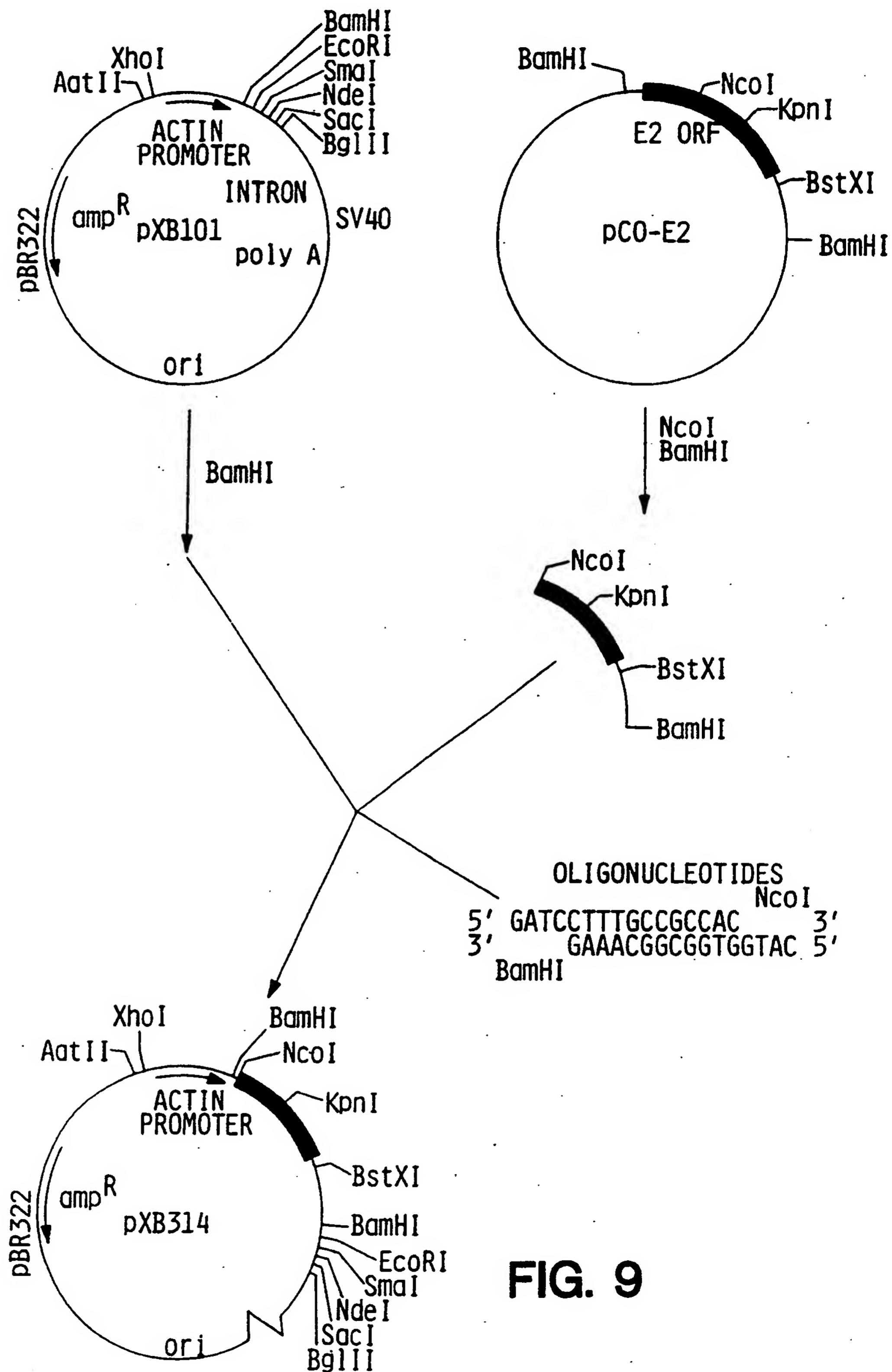


FIG. 9

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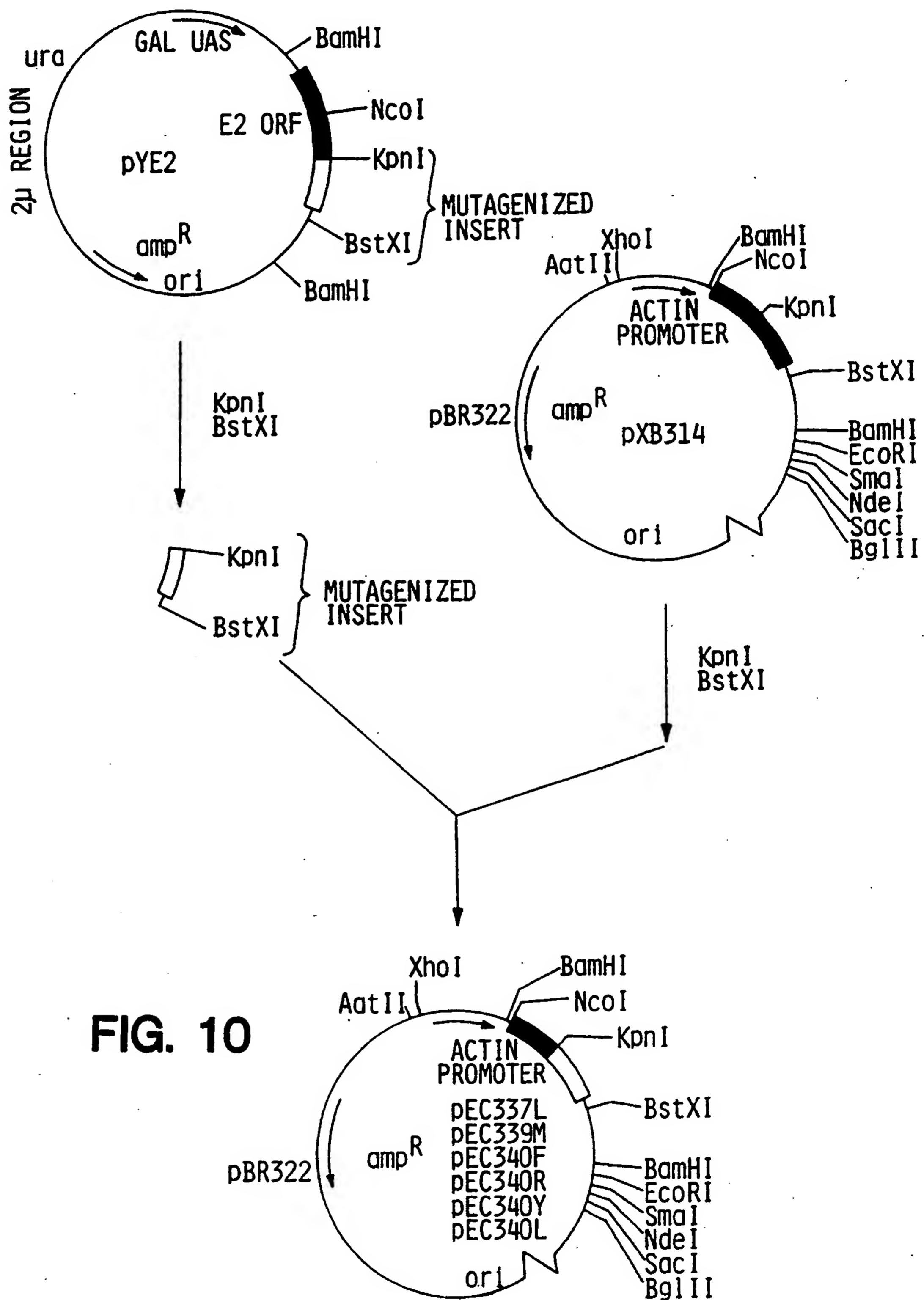
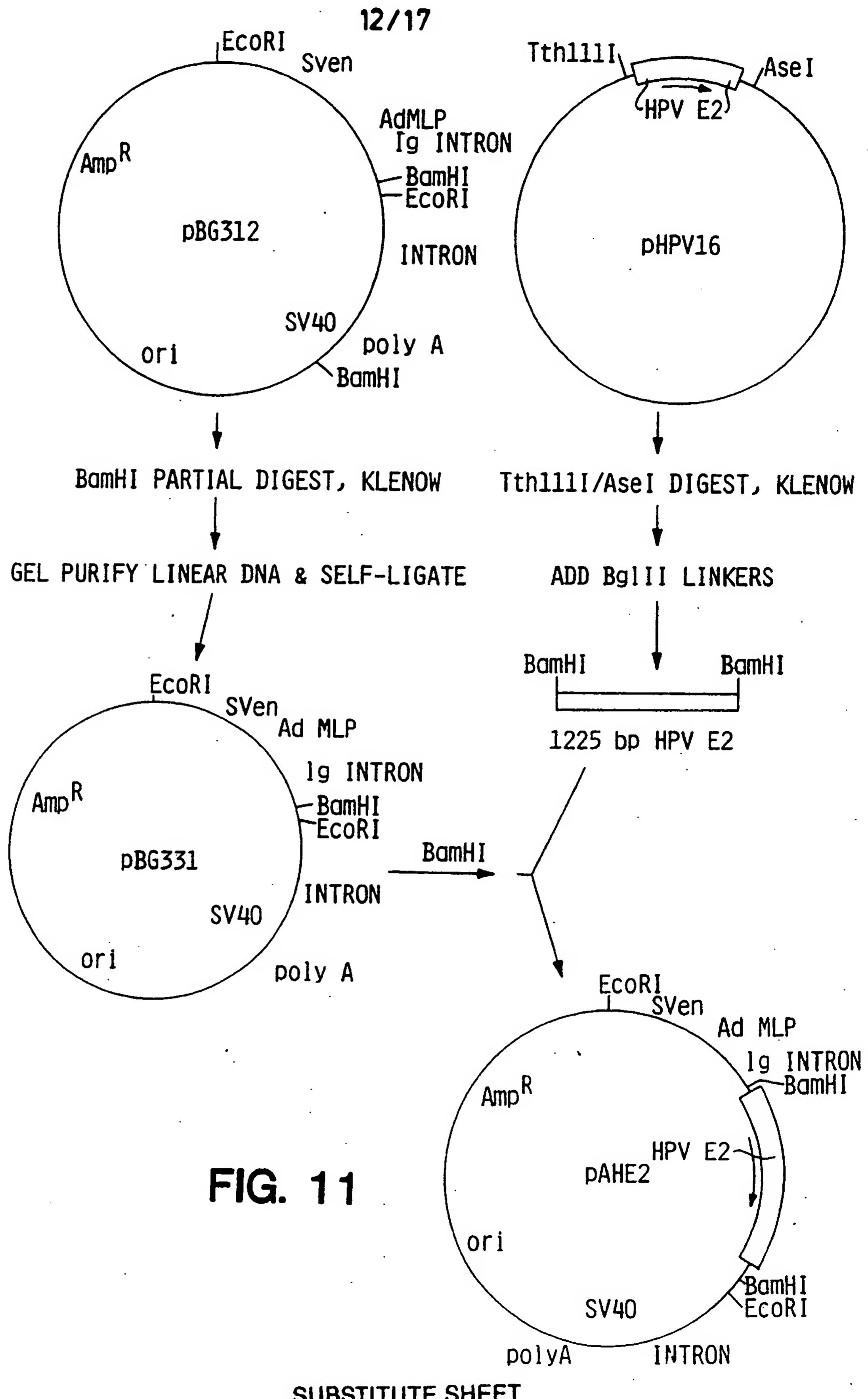


FIG. 10



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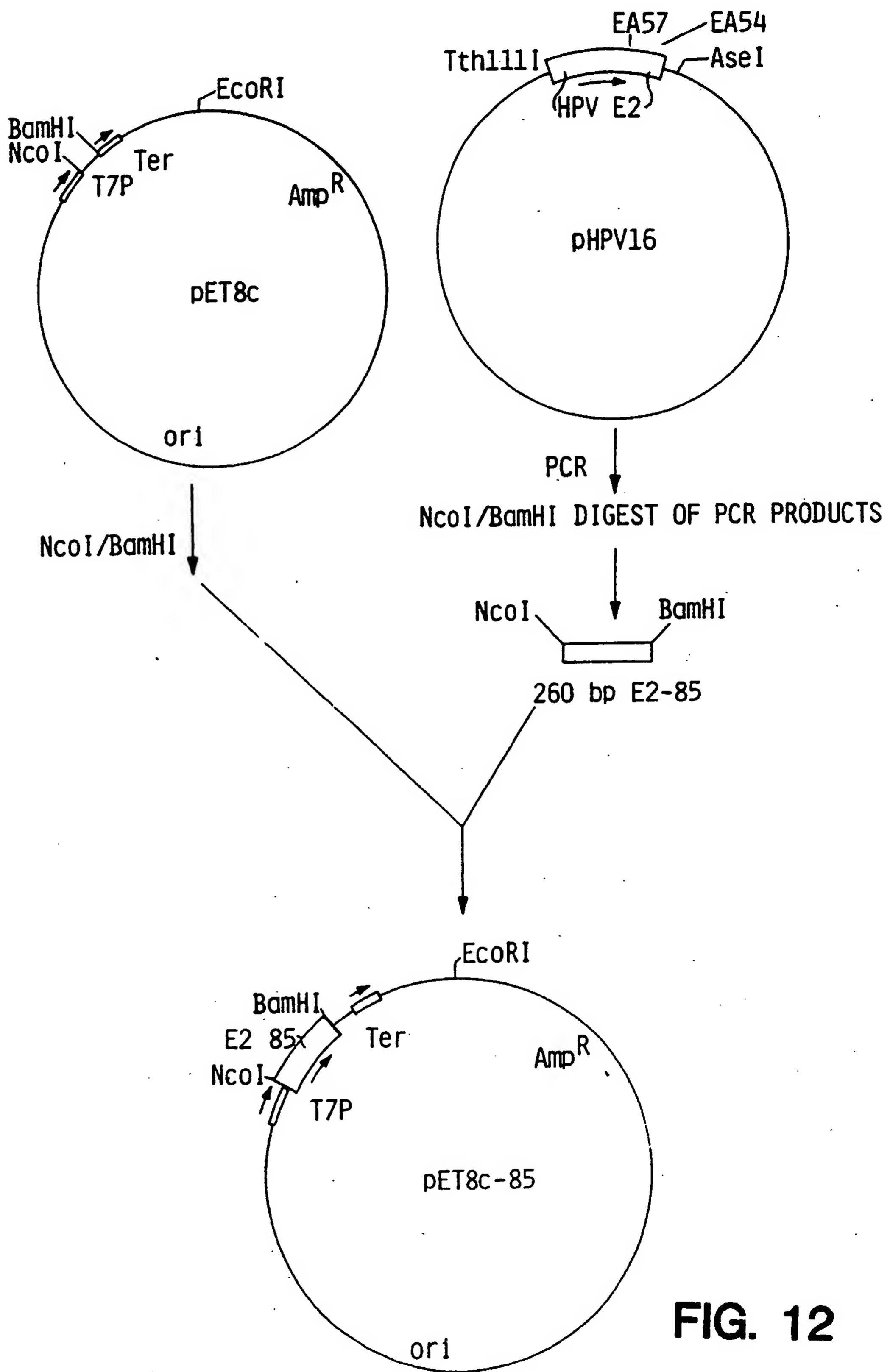
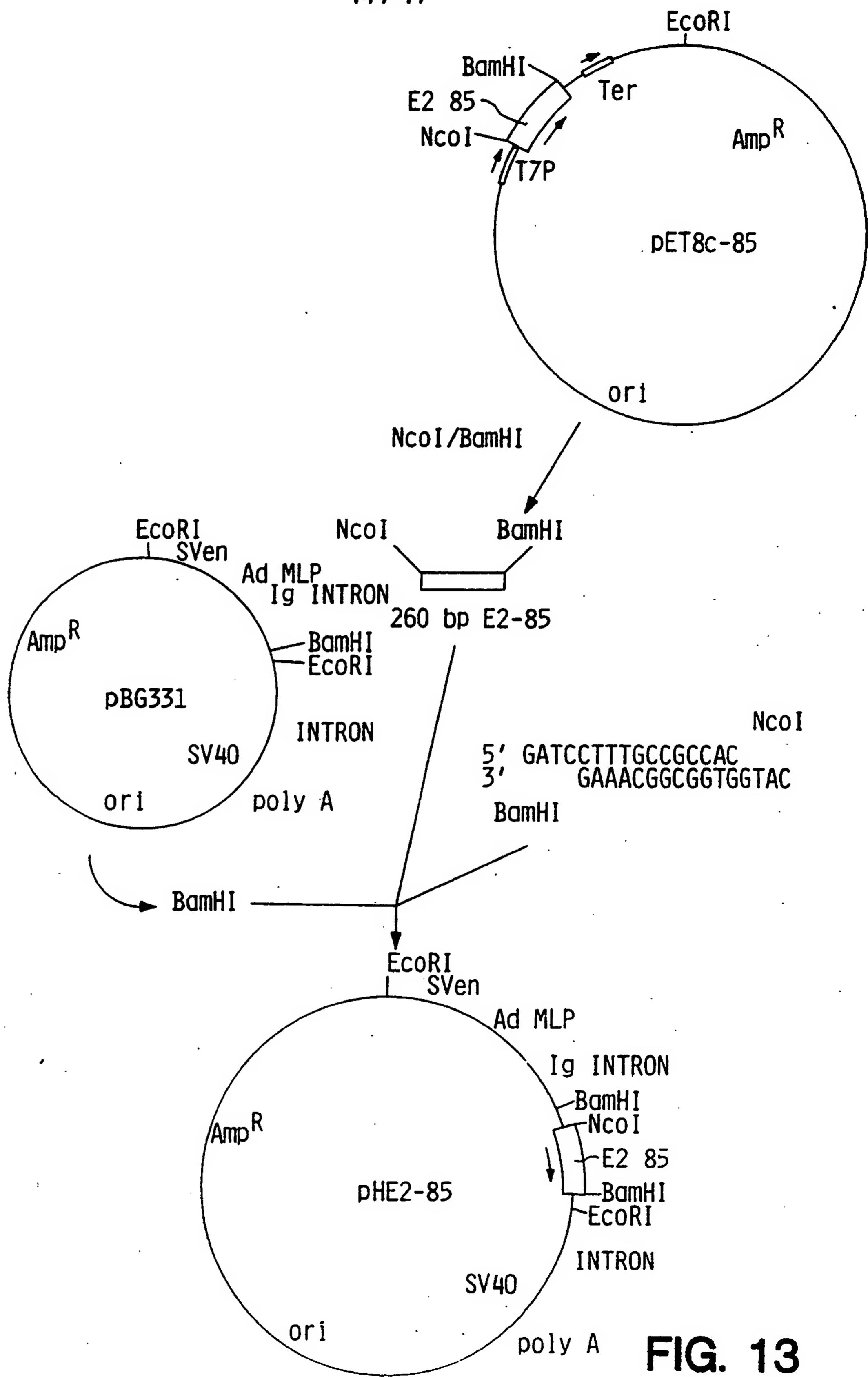


FIG. 12

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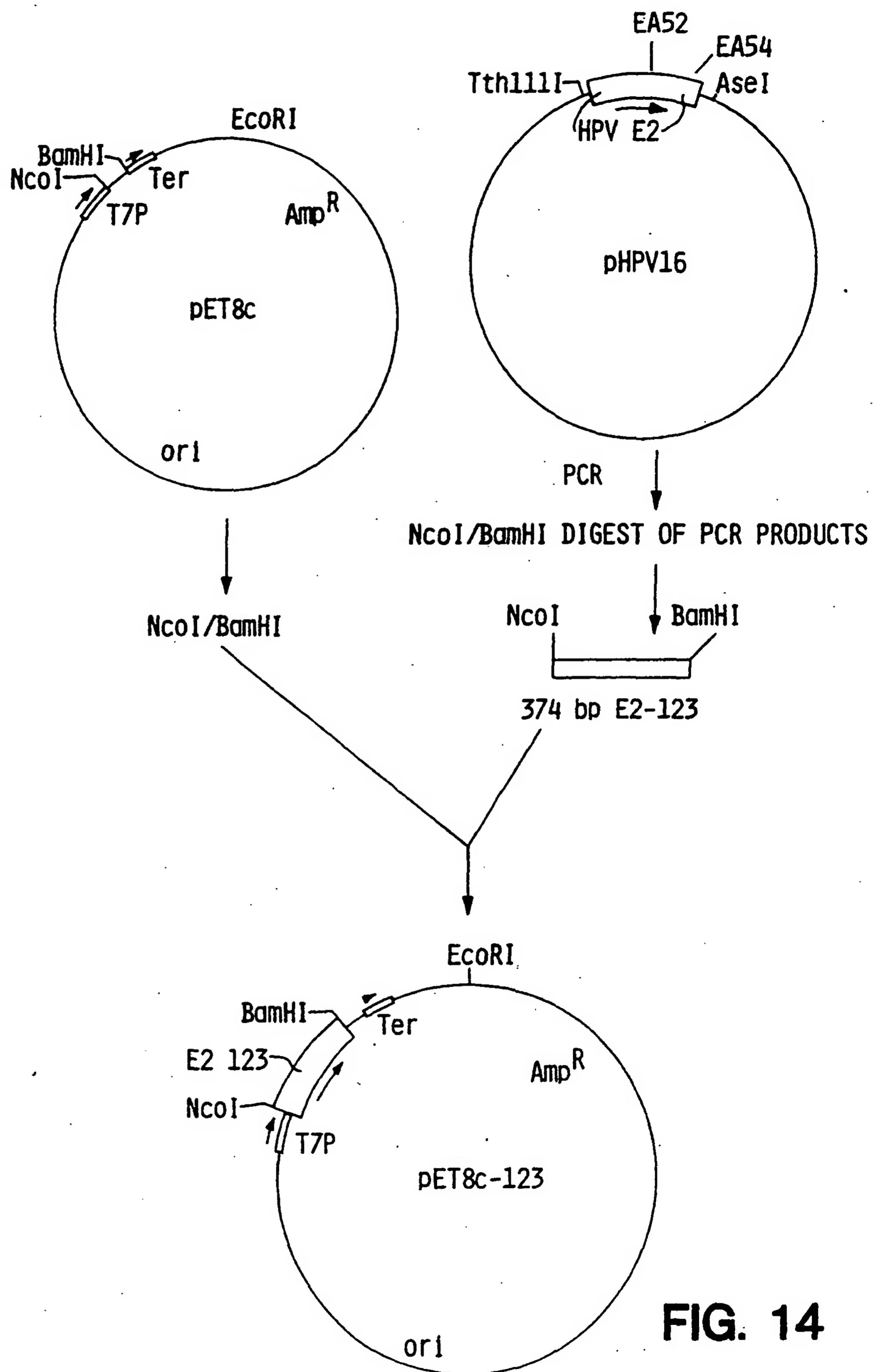


FIG. 14

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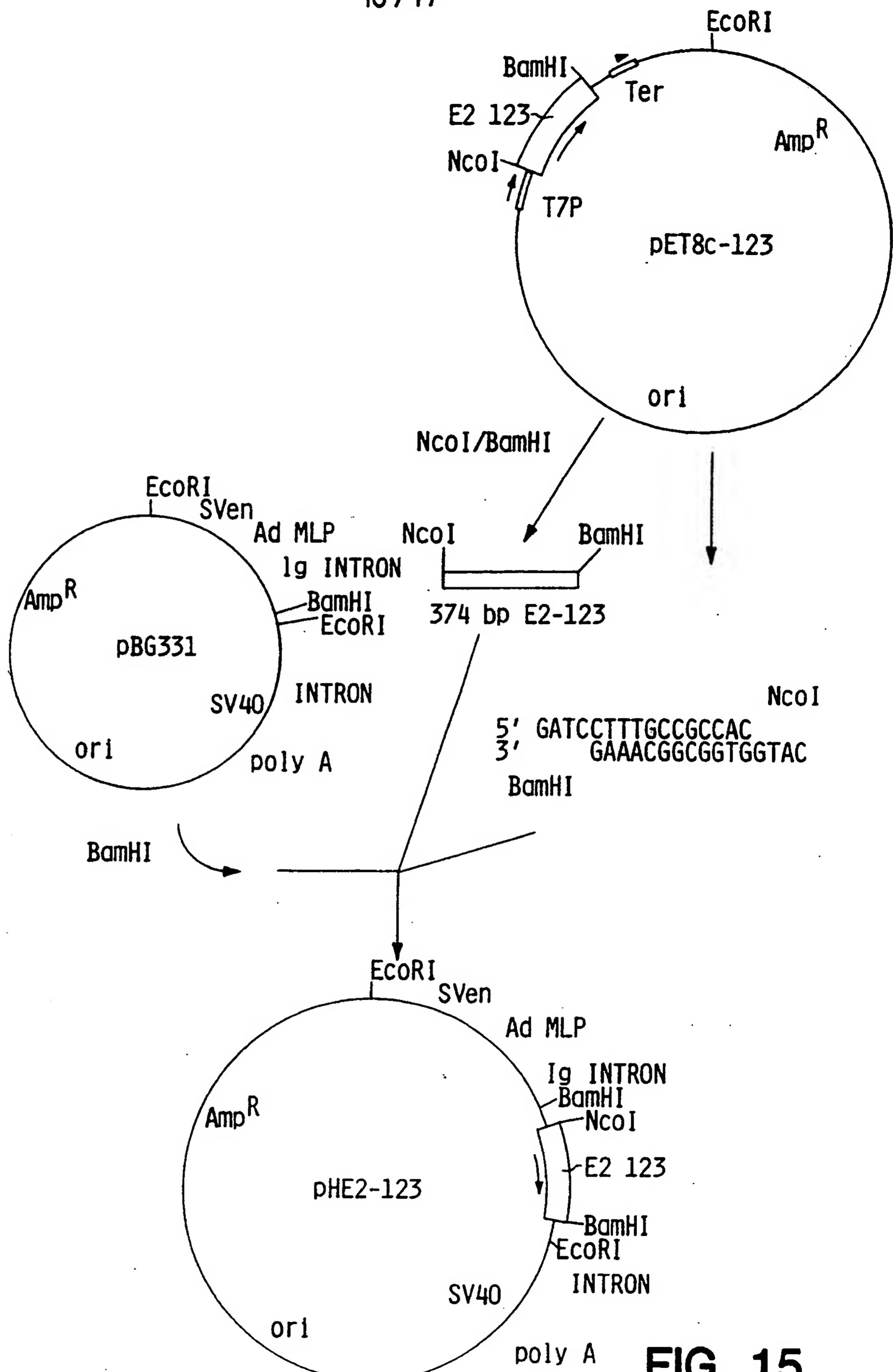


FIG. 15

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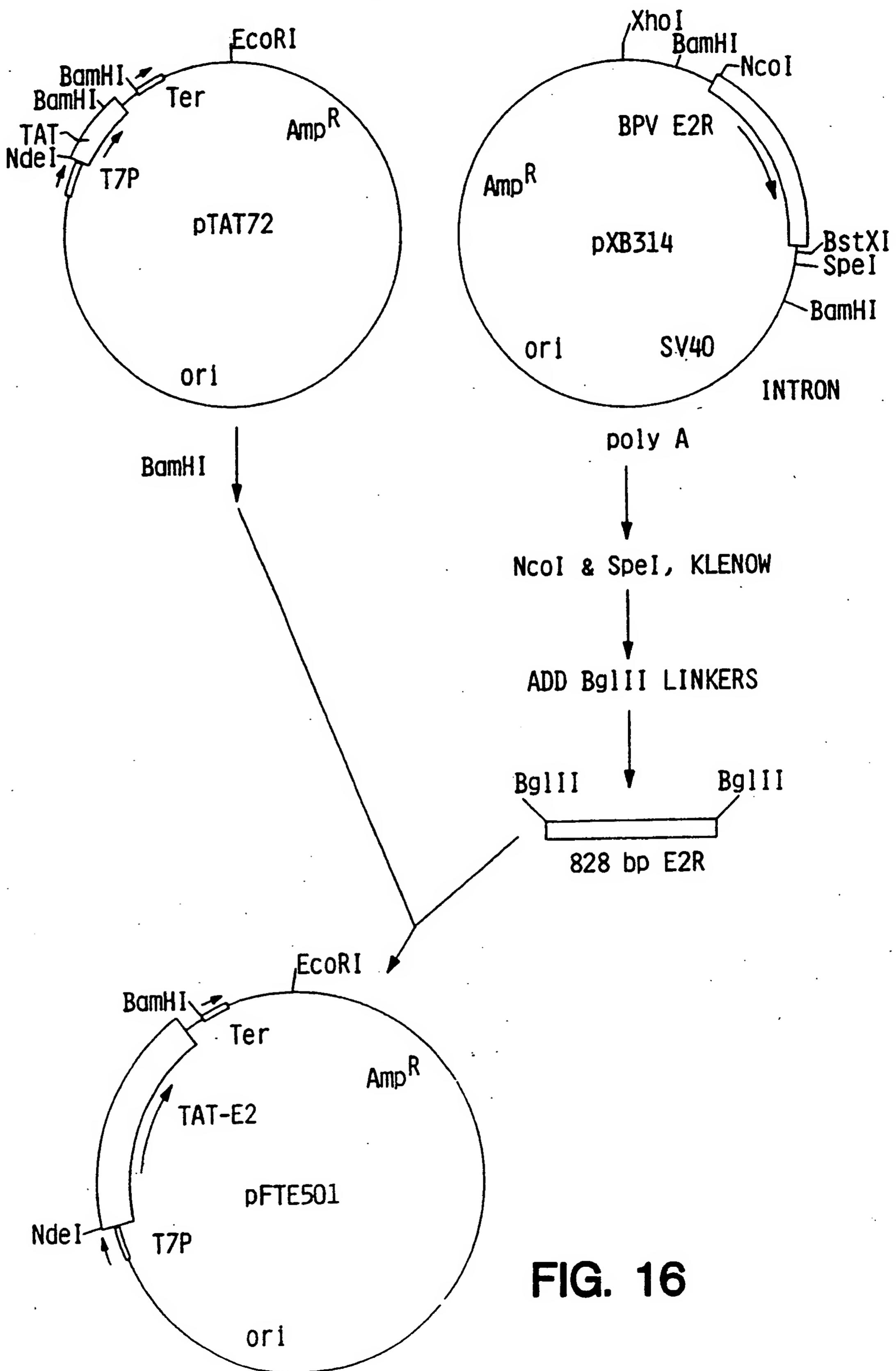


FIG. 16

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 92/00652

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1.5	C 12 N 15/37	C 07 K 13/00	C 12 N 15/62
C 12 N 1/00	C 12 N 1/21	A 61 K 37/02	//(C 12 N 1/21)
C 12 R 1:19)			

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols	
Int.C1.5	C 12 N	C 07 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	Genes and Development; vol. 6, no. 1, January 1992, S.S, PRAKASH et al.: "Amino acids necessary for DNA contact and dimerization imply novel motifs in the papillomavirus E2 trans-activator", pages 105-116, see the whole document ---	1-6,8-16,18
A	WO,A,8912461 (ST. LOUIS UNIVERSITY) 28 December 1989, see the whole document -----	1

¹⁰ Special categories of cited documents :^{"A"} document defining the general state of the art which is not considered to be of particular relevance^{"E"} earlier document but published on or after the international filing date^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)^{"O"} document referring to an oral disclosure, use, exhibition or other means^{"P"} document published prior to the international filing date but later than the priority date claimed^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.^{"&"} document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

25-05-1992

Date of Mailing of this International Search Report

15.07.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer